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XXXVII. THE INFLUENCE OF FREE FATTY ACIDS IN THE INTESTINAL CONTENTS ON THE EXCRETION OF CALCIUM AND PHOSPHORUS.

By STEPHEN VEITCH TELFER.

From the Institute of Physiology, University of Glasgow, and the Royal Hospital for Sick Children, Glasgow.

(*Received April 12th, 1921.*)

WITHIN recent years a considerable amount of work has been done on the changes in the metabolism of calcium and phosphorus in infants, especially in relation to the pathological processes which directly involve the skeletal system. Part of this work has been concerned with the study of the effects of fat in the diet on the retention of these elements.

The results of Orgler, Meyer, Steinitz, Rothberg and Freund, quoted by Findlay [1918] in his survey of rickets seemed to indicate that there is a close relationship between the nature of the fat in the diet and the degree of calcium retention. These workers found a much greater retention with cod-liver oil and sesame oil than with butter fat. Their results, however, have not received full confirmation from later investigations. The work of Holt, Courtney and Fales [1920] has also served to direct attention to the relationship between calcium absorption and fat digestion. These workers found that in infants "the best absorption of calcium was obtained when the calcium intake bore a definite relation to the fat intake." Bosworth *et al.* [1918] have concluded from their observations that impaired fat digestion in infants, artificially fed, is due to the excessive quantity of calcium which cow's milk contains. These workers hold that the calcium soaps formed in the intestine occlude a certain quantity of neutral fat and free fatty acids which thus escape absorption. The exact nature of the relation between fat digestion and calcium retention is, however, still undetermined.

During the course of a series of observations on rachitic children, some information was particularly desired of the influence of the fatty acids set free in digestion on the degree of retention and mode of excretion of calcium and phosphorus. To obtain this, it was obviously important to compare the excretion of calcium and phosphorus in normal digestion with their excretion when a large excess of free fatty acids persisted unabsorbed in the intestine.

It was shown by Nöel Paton and Balfour [1891] in their study of the fat absorption in a case of biliary fistula in a woman, that the absence of bile from the gut interferes considerably with the absorption of the products of fat

digestion. Their results agreed with those of Munk and other previous observers who worked on dogs with complete biliary fistulae.

Hutchison and Fleming [1920] in a recent paper have also shown, from observations on a case of congenital biliary atresia, that the exclusion of bile from the gut is associated with a large and persistent excess of free fatty acids in the intestinal contents.

The mode of excretion of calcium and phosphorus under the conditions in which bile was completely or even partially absent from the intestine was therefore thought to be worthy of consideration, as some light might be thrown upon the relation of fats to calcium under normal and pathological conditions.

The following are the details of studies made in two cases of congenital biliary atresia, with complete exclusion of bile from the gut, and in one of jaundice in an infant suffering from syphilitic hepatitis, with partial exclusion of bile. The analytical data of these three cases are compared with those obtained in the examination of a normal infant.

Methods.

The subjects were placed on a "metabolism" bed, devised by Findlay, so that the urine and faeces could be collected separately and quantitatively. The faeces were dried on a steam-bath till constant in weight, then finely ground, or if pasty, thoroughly mixed in a mortar and preserved in air-tight bottles. Each daily quantity of urine was measured, acidified with nitric acid, and the bulk diluted to one litre. The diet in all cases consisted of fresh cow's milk containing approximately 3 % fat.

The calcium and phosphorus were determined in the ash of the dried faeces by the usual gravimetric methods. The neutral fat and free fatty acids were extracted directly from the dried faeces with ether. The combined fatty acids were obtained by splitting the soaps in the residue with hydrochloric acid and extracting the liberated fatty acids in a separating funnel.

Results.

In Table I the daily amounts of urine and dried faeces are recorded, together with the quantities of CaO and P_2O_5 which these contained. In Table II, which shows the distribution of the fat in the dried faeces, the values given for free fatty acids were obtained by dissolving the ether extract (containing fat and free fatty acids) in neutral alcohol, titrating the free acids with $N/10$ NaOH, and calculating to oleic acid. The neutral fat was found by difference. In Table III the values recorded for CaO existing as soaps in the dried faeces were calculated from the weight of combined fatty acids found by experiment. An experimental determination of the mean molecular weight of the insoluble fatty acids of butter fat combining with calcium was found to give the value 274, and the coefficient $\frac{28}{274}$ or 0·102, was therefore used in calculating the percentage of CaO existing as soap from the weight of combined

fatty acids found. The difference between the total CaO and that existing as insoluble soap was taken as the CaO existing as phosphate of calcium.

CASE 1. NORMAL SUBJECT. J. H., AET. 4 MONTHS.

Intake: 720 *cc. cow's milk daily.*

Table I.

Day	Urine cc.	Faeces (dry) g.	CaO Urine g.	CaO Faeces g.	P_2O_5 Urine g.	P_2O_5 Faeces g.
1	350	10·8	0·016	1·18	0·44	0·83
2	440	10·7	0·016	1·07	0·57	0·92
3	350	10·2	0·016	1·03	0·49	0·78
4	390	11·7	0·02	0·95	0·57	0·73

Table II. *Distribution of fat in dried faeces* (%).

Day	Neutral fat	Free fatty acids (as oleic acid)	Combined fatty acids	Total fat
1	1·07	11·13	19·15	31·35
2	1·81	10·99	16·35	29·15
3	1·62	10·43	17·90	29·95
4	6·08	12·12	12·20	30·40

Table III. *Distribution of CaO and* P_2O_5 *in dried faeces* (%).

Day	CaO (total)	CaO (as soaps)	CaO (as phosphate)	P_2O_5 (total)	% CaO as soaps of total CaO
1	10·9	1·96	8·94	7·7	17·9
2	10·0	1·66	8·34	8·6	16·6
3	10·1	1·82	8·28	7·7	18·0
4	8·1	1·24	6·86	6·2	15·3

CASE 2. CONGENITAL BILIARY ATRESIA. J. S. (MALE), AET. 3 MONTHS.

Intake: 720 *cc. cow's milk daily.*

Table I.

Day	Urine cc.	Faeces (dry) g.	CaO Urine g.	CaO Faeces g.	P_2O_5 Urine g.	P_2O_5 Faeces g.
1	310	28·0	0·036	1·14	0·91	0·4
2	430	24·4	0·038	0·936	0·87	0·34
3	420	23·4	0·046	0·88	0·95	0·28
4	420	30·5	0·048	0·956	0·99	0·26

Table II. *Distribution of fat in dried faeces* (%).

Day	Neutral fat	Free fatty acids (as oleic acid)	Combined fatty acids	Total fat
1	17·47	33·13	25·75	76·35
2	14·44	34·26	25·45	74·15
3	12 63	31·72	30·15	74·53
4	8·2	40·6	29·35	78·15

Table III. *Distribution of CaO and P_2O_5 in dried faeces* (%).

Day	CaO (total)	CaO (as soaps)	CaO (as phosphate)	P_2O_5 (total)	% CaO as soaps of total CaO
1	4·07	2·63	1·44	1·42	64·6
2	3·83	2·60	1·23	1·35	67·8
3	3·76	3·08	0·68	1·19	81·9
4	3·76	2·99	0·77	0·85	79·5

CASE 3. JAUNDICE IN SYPHILITIC HEPATITIS. R. W. (MALE), AET. 17 WEEKS.

Intake: 300 *cc. cow's milk daily.*

Table I.

Day	Urine cc.	Faeces g.	CaO Urine g.	CaO Faeces g.	P_2O_5 Urine g.	P_2O_5 Faeces g.
1	90	9·0	trace	0·387	0·321	0·231
2	78	7·6	trace	0·281	0·228	0·174

Table II. *Distribution of fat in dried faeces* (%).

Day	Neutral fat	Free fatty acids (as oleic acid)	Combined fatty acids	Total fat
1	15·6	29·9	25·05	70·55
2	12·63	29·32	29·75	71·70

Table III. *Distribution of CaO and P_2O_5 in dried faeces* (%).

Day	CaO (total)	CaO (as soaps)	CaO (as phosphate)	P_2O_5 (total)	% CaO as soaps of total CaO
1	4·3	2·56	1·74	2·59	59·5
2	3·7	3·04	0·66	2·3	82·1

DISCUSSION.

The most striking difference between the faeces of the normal subject and those in which bile was partly or completely absent from the gut lies in the fat content. Hutchison [1920] showed that approximately one-third of the dried faeces of a normal child consists of a fat and fatty acids. The results recorded in Table II of the series demonstrate that the normal fat content is increased by the absence of bile from the gut. In the case of congenital biliary atresia (Case 2), no bile was present in the faeces, but it was not completely absent in Case 3. In both, although a considerable amount of fat splitting had occurred, the absorption of fat had fallen markedly from the normal, and over 70 % of the dried faeces consisted of fatty derivatives in each case. These results confirm the findings of Hutchison and Fleming in their case of congenital biliary atresia.

The presence of this excess of fat, free fatty acids and insoluble soaps must naturally influence the bulk of the faeces excreted per diem. The naked eye appearance of these is characteristic. They are pale in colour and of a pasty

consistency. During the process of drying on a steam-bath they partly melt. Even when water-free they remain quite soft, and in marked contrast to dried normal faeces, which can be readily ground to a fine powder. Care has to be exercised in mixing such faeces so that uniform samples may be obtained for examination.

The weight of dried faeces daily obtained in the case of congenital biliary atresia (Case 2) is seen to be more than double that in the normal child of the same age. The amounts of cow's milk ingested were almost identical in the two cases. This increase in weight is evidently due to the presence of the excess of fatty derivatives. It will be observed that the weight of dried faeces per diem of the infant of 17 days suffering from jaundice does not fall much below that of the normal child of three months. Correlated with the presence of an excess of fatty derivatives (Cases 2 and 3), there is a diminution in the percentage of CaO and P_2O_5 as compared with the normal. This must be due largely to "dilution" by the fat, since it was found that there was no increased retention of calcium and phosphorus. The significance of the abnormally low percentage of phosphoric acid will be discussed later. In a series of analyses of the normal stools of infants on a diet of fresh cow's milk the calcium content of the dried faeces was found to vary only between very narrow limits and was generally about 10 % CaO. The CaO content of the faeces depends to a very large degree on the percentage of calcium in the diet. When the latter is increased there is a corresponding increase of calcium in the faeces. The results of Berg [1911], who added quantities of different calcium salts to his own diet and found that almost all the added calcium appeared in the faeces, demonstrate this clearly. Since the calcium content of cow's milk is very constant, the small percentage of CaO found in the dried faeces in Cases 2 and 3, must be due chiefly to the increased excretion of fat and fatty acids.

The manner in which the normal distribution of calcium in the dried faeces is altered by the presence of an excess of free fatty acids in the gut is evident from a comparison of the results in Table III of the series. The faeces of the normal subject contained less than 20 % CaO as soap, the remainder of the CaO existing as phosphate. In Case 2 the urine contained regularly more than twice the amount of P_2O_5 present in the faeces. The increased elimination of P_2O_5 in the urine is also evident, though to a less degree, in the case of jaundice (Case 3). In all three the intakes of calcium and phosphorus were in excess of the amounts found in the excreta, so that there was a positive retention of both elements.

The deviation of the greater part of the phosphoric acid from the normal excretory route is probably related to the fixation of calcium as soaps. This condition again is evidently dependent on the persistence of an excess of free fatty acids in the intestine, the non-absorption of these being due to the absence of bile. There has been no marked interference with fat digestion, in the two pathological cases. Even in the case of biliary atresia, in which bile was completely absent from the intestinal contents, fat splitting has been fairly

complete. Also, it is possible as Bosworth *et al.* [1918] have stated, that the presence of some neutral fat in the stools is partly due at least to mechanical occlusion of the fat in the mass of insoluble soaps. The increased amount of neutral fat in the stools of Cases 1 and 2 may have been dependent on this cause. The appearance of neutral fat in comparatively small excess of the normal in faeces consisting largely of insoluble soap, need not necessarily imply a defect in fat splitting.

It is of interest to note that Schabad [1910] in a series of observations on rachitic children, found a diminished excretion of phosphoric acid by the urine during the active stages of the disease, the increased elimination occurring by the faeces.

As the changes found in the mode of excretion of calcium and phosphorus appeared to be related to the persistence of an excess of free fatty acids in the intestine, the distribution of these elements in the excreta when the amount of fat ingested was varied seemed worthy of study. Further observations were therefore made on a case of congenital biliary atresia in a female infant (Case 4) with a view to ascertaining the effects of increasing concentration of fatty acids in the gut on the excretion of the two elements. The collection of the total urine and faeces quantitatively was found to be impossible in this case. The difficulty of obtaining specimens of faeces uncontaminated by urine could only be overcome by catheterisation before the bowel was evacuated. During an initial period of three days the milk given contained 3 % fat. Separated cow's milk, with the fat content reduced to 0·8 % fat was then substituted for ordinary milk over a succeeding period of three days. Finally an artificially prepared cream (5·3 % fat) was given during the last period. A sample of faeces at the close of each of these intervals was taken for analyses. The following results were obtained.

Case 4. Congenital Biliary Atresia. J. Sy (female), aet. 3½ months.

Intake: 720 *cc. cow's milk daily.*

Table I. *Distribution of fat in dried faeces* (%).

	Period 2. 0·8 % fat	Period 1. 3·0 % fat	Period 3. 5·3 % fat
Neutral fat	10·68	5·50	15·26
Free fatty acids (as oleic acid)	25·34	39·29	48·43
Combined fatty acids ...	28·44	33·23	17·17
Total	64·46	78·02	80·86

Table II. *Distribution of CaO and* P_2O_5 *in dried faeces* (%).

	Period 2. 0·8 % fat	Period 1. 3·0 % fat	Period 3. 5·3 % fat
CaO (total)	8·8	5·37	2·12
CaO (as soaps)...	2·9	3·38	1·75
CaO (as phosphate)	5·9	1·99	0·37
P_2O_5 (total)	4·89	1·97	0·65
% CaO as soaps of total CaO ...	32·9	62·9	82·5

The abnormally high excretion of fat is again evident in this case of biliary atresia in which bile was totally absent from the gut. With increase in the fat content of the ingested milk there is an increase of fatty derivatives (Table I) in the faeces and a reduction of the mineral elements. The diminution of the phosphorus content is greater, however, than that of the calcium, which indicates that more phosphoric acid is being removed from combination with calcium as the concentration of fatty acids in the intestinal contents increases.

The increase of calcium soaps and the diminution of phosphoric acid (Table II) in the faeces is evidently correlated with the increased concentration of fat in the diet. Since the degree of absorption of the fatty acids in the absence of bile from the gut has presumably remained at the same low value throughout the three periods, it must be concluded that the changes in the mode of excretion of calcium and phosphorus observed are dependent on the concentration of free fatty acids in the intestinal contents.

In view of these results an explanation of the changes which the calcium and phosphorus of the foodstuff have undergone might be attempted. Calcium is present in cow's milk partly in organic combination, partly as soluble salts, and partly as phosphate. In the presence of the gastric juice the greater part of both the calcium and phosphorus passes into solution. No precipitation of these elements in insoluble form is possible at this stage owing to the presence of free acid. With the passage of the acid gastric contents into the duodenum neutralisation will begin as a result of contact with the alkaline pancreatic secretion and bile. As the reaction of the intestinal contents proceeds towards alkalinity prior to the onset of tryptic digestion the precipitation of insoluble phosphate of calcium before the commencement of fat hydrolysis will take place. In the normal subject a large proportion of the calcium and phosphorus is therefore limited to the gut in insoluble form and as such appears in the faeces. The existence of 80–90 % of the total CaO as phosphate in normal dried faeces lends support to the view. The small proportion of calcium which appears as soaps in normal faeces may be derived possibly from the calcium in combination with casein which undergoes disintegration after the onset of tryptic digestion.

The amount of phosphorus in cow's milk is in excess of the equivalent amount required to combine with all the calcium to form tricalcium phosphate. Although normally a considerable proportion of the calcium may be fixed in insoluble form as calcium phosphate, it does not necessarily follow that the latter will remain unacted upon by the products of digestion. Tri-calcium phosphate is soluble in the lower fatty acids and it can be shown by experiment *in vitro* that precipitated calcium phosphate is redissolved in an excess of the volatile water-soluble fatty acids of butter fat. The latter consist almost entirely of butyric and caproic acids. If the free fatty acids of fat hydrolysis persisted in excess in the intestine, as in the two pathological cases under discussion, the calcium phosphate, if initially precipitated, would tend to pass into solution. In the presence of the higher fatty acids and their soluble soaps

a secondary reaction may then result in the formation of insoluble calcium soaps and the liberation of phosphoric acid.

The calcium salts of the lower fatty acids are soluble and in this state calcium may be absorbed, as suggested by Bosworth *et al.* The phosphoric acid, which has been liberated from combination with calcium phosphate as a result of the precipitation of the calcium as soaps, may then be absorbed as it exists in soluble form. The increased elimination of phosphoric acid by the urine, as found in the two pathological cases, would naturally follow this increased absorption from the intestinal tract.

Summary.

1. Biliary atresia and obstructive jaundice afford a means of studying the effect of non-absorption of fatty acids on the distribution of calcium and phosphorus in the faeces and urine and on the absorption of these elements.

2. In complete exclusion of bile from the gut over 70 % of the dried faeces may consist of fatty derivatives.

3. The non-absorption and persistence of an excess of fatty acids in the intestine is associated with a modification of the normal excretion of calcium and phosphorus. The calcium is found in the faeces as soap in large excess of the normal. The phosphorus, which normally is excreted by the bowel as tricalcium phosphate, is liberated from combination with calcium and is absorbed and eliminated by the urine.

4. The degree to which the normal excretion of calcium and phosphorus can thus be varied is shown to depend on the concentration of the free fatty acids in the intestinal contents.

This work was carried out in connection with the Child Life Investigation of the Medical Research Council. I desire to express my indebtedness to Prof. D. Nöel Paton for much helpful advice and criticism.

REFERENCES.

Berg (1911). *Biochem. Zeitsch.* **30**, 139.
Bosworth, Bowditch and Giblin (1918). *Amer. J. Dis. Child.* **15**, 397.
Findlay (1918). *Med. Res. Comm. Spec. Rep.* Series **20**, 31
Holt, Courtney and Fales (1920). *Amer. J. Dis. Child.* **19**, 201.
Hutchison (1920). *Quart. J. Med.* April, **13**, 277.
Hutchison and Fleming (1920). *Glasgow Med. J.* **12**, 65.
Paton and Balfour (1891). *Lab. Rep. Roy. Coll. Phys. Edin.* No. 3, 191.
Schabad (1910). *Arch. Kinderh.* **54**, 85.

XXXVIII. THE EFFECTS OF AN UNBALANCED DIET IN THE PRODUCTION OF GUINEA-PIG SCURVY.

By GEORGE MARSHALL FINDLAY.

From the Royal College of Physicians' Laboratory, Edinburgh.

(*Received April 13th, 1921.*)

It has long been recognised that when pigeons are fed on polished rice the addition of an excess of carbohydrates to the diet leads to a more rapid onset of the symptoms of beriberi. According to Funk [1914] the addition of an excess of any of the basal foodstuffs has some effect in hastening the appearance of beriberi, the effect being most marked with polysaccharides and disaccharides, least marked with fats, while proteins occupy an intermediate position. In the following experiments an attempt has been made to ascertain whether the same conditions hold good when guinea-pigs are deprived of vitamin C.

Twenty-four guinea-pigs were divided into four equal groups, each group consisting of three males and three females. Group A received a basal diet of oats and bran *ad libitum* with the addition of 60 cc. of autoclaved milk per diem—Table I. Group B received the same diet with the addition of 10 grams of caseinogen, the caseinogen before administration being autoclaved at 120° for two hours to destroy any trace of the anti-scorbutic factor—Table II. Group C received the same basal diet as group A with the addition of 10 grams of cane sugar a day—Table III—while in group D the cane sugar was replaced by a daily ration of 10 cc. of cod-liver oil—Table IV.

In avian beriberi the onset of symptoms is, as a rule, well marked, but in guinea-pig scurvy it is much more gradual. By feeding the animals oneself and by carefully handling them, it is however quite possible to ascertain with considerable accuracy the date at which haemorrhages first occur in the region of the joints. The date at which the first symptoms appear is not materially affected by an excess of protein, carbohydrate or fat in the diet. The date of death is, however, slightly accelerated by an excess of protein and carbohydrate in the diet, the acceleration being less marked when fats are in excess. The most striking feature revealed was the considerable loss of body weight in the guinea-pigs fed with excess of carbohydrate, this loss being equivalent to a loss of 7·26 grams per day per guinea-pig, whereas animals fed on the oats, bran, milk diet only lost 2·64 grams per day.

Table of Results.

I.

Number	Weight at beginning of experiment g.	Weight at end of experiment g.	Percentage loss in weight	Day of first symptoms	Day of death
Guinea-pigs on a basal diet of oats and bran *ad libitum* and 60 cc. of autoclaved milk per diem.					
1	252	218	13·5	16	27
2	374	268	28·3	13	33
3	468	401	14·3	15	41
4	250	210	16·0	14	25
5	440	257	41·5	13	27
6	282	239	15·2	15	26
Average	344·3	265·5	21·5	14·3	29·8

II.

Guinea-pigs on a basal diet of oats and bran *ad libitum* and 60 cc. of autoclaved milk per diem plus 10 g. of caseinogen.

7	302	255	15·5	13	27
8	310	262	15·5	12	14
9	248	205	17·3	15	21
10	340	275	19·1	15	25
11	262	196	29·0	14	26
12	273	224	17·9	13	27
Average	289·2	236·2	19·5	13·6	23·3

III.

Guinea-pigs on a basal diet of oats and bran *ad libitum* and 60 cc. of autoclaved milk per diem plus 10 g. of cane sugar.

13	465	298	35·9	16	29
14	466	304	34·8	13	24
15	498	320	35·7	12	22
16	428	318	25·7	15	23
17	470	295	37·3	14	20
18	520	284	45·3	13	23
Average	474·5	303·2	35·8	13·8	23·5

IV.

Guinea-pigs on a basal diet of oats and bran *ad libitum* and 60 cc. of autoclaved milk per diem plus 10 cc. of cod-liver oil.

19	298	250	16·2	14	21
20	230	191	17·1	13	19
21	263	224	15·0	13	22
22	228	178	22·1	15	32
23	319	253	20·8	14	31
24	350	281	19·6	16	27
Average	281·3	229·5	18·5	14·2	25·3

It is of some interest to note that the histological appearances found in the bones and joints of the animals which were given cod-liver oil were identical with those met with in other cases of acute guinea-pig scurvy, thus showing once again that the lack of vitamin A is in no way responsible for the lesions that occur in experimental scurvy.

Conclusions.

1. Guinea-pigs fed on a diet lacking in vitamin C do not develop scurvy more rapidly when the diet contains an excess of either protein, fat or carbohydrate.

2. Death is slightly accelerated in guinea-pigs suffering from scurvy when the diet contains an excess of either protein or carbohydrate.

3. The loss of weight is greatly increased in guinea-pigs suffering from scurvy when the diet contains an excess of carbohydrate.

REFERENCE.

Funk (1914) *Zeitsch. physiol. Chem.* **89**, 378.

XXXIX. NOTE ON THE OXYGEN CONSUMPTION OF AMPHIBIAN MUSCLE AND NERVE.

By NEIL KENSINGTON ADAM[1].

From the Biochemical Laboratory, Cambridge.

(*Received April 14th, 1921.*)

Some observations made in 1919 with the instrument described in a recent paper [1920] showed clearly the course of oxygen consumption in excised muscle and nerve in an atmosphere of nearly pure (95 %) oxygen, when the tissues were at rest, and the different effects of electric stimulation on the two tissues. Although these experiments were incomplete, and obviously capable of much improvement, yet it seems desirable to record such results as were obtained now, since circumstances have arisen which seem likely to prevent my continuing research in this subject.

The rates of respiration observed in resting tissues were the following. Sartorius muscles, 0·10 to 0·135 cc. per g. per hour, reduced to N.T.P.; gastrocnemius muscles, 0·05 to 0·12 cc. per g. per hour; sciatic nerves, 0·05 to 0·08 cc. per g. per hour. The number of experiments done was not enough to determine whether any seasonal change occurs in the respiratory activity of these tissues.

In muscles the rate of respiration observed was usually somewhat higher than this for the first hour or so after excision, and during this preliminary period the rate fell off, reaching finally a steady value. At the end of this preliminary period, which was probably due to the recovery of the tissue from the stimulatory effects of accidental damage during the dissection, the tissue settled down to a constant rate of respiration, which was maintained for about twenty-four hours. This appeared to be the normal resting condition. Subsequently a rise usually commenced, and the rate of respiration observed accelerated continuously till it reached a value many times greater than the steady rate. This rise was obviously due to bacterial action. The general course of survival oxygen consumption thus follows Fletcher's curve [1898] for carbon dioxide output very closely.

During the period of constant respiration the exactness with which the rate was maintained was very remarkable (Fig. 1). Although variations as small as 1 % could easily have been detected from hour to hour, yet the rate was very frequently found constant within the error of reading the scale for many hours

[1] This work was carried out during the tenure of the Benn W. Levy studentship.

consecutively. It should be mentioned that irregular divergences from the steady rate were observed occasionally, but when these were most marked the tissue was generally found to have its response to an electric stimulus considerably impaired. Unfortunately the risk of damage to the tissue in the experiments was greater than is necessary, since an attempt was made to tie the very short tendons at the upper end of the muscle: it would be preferable

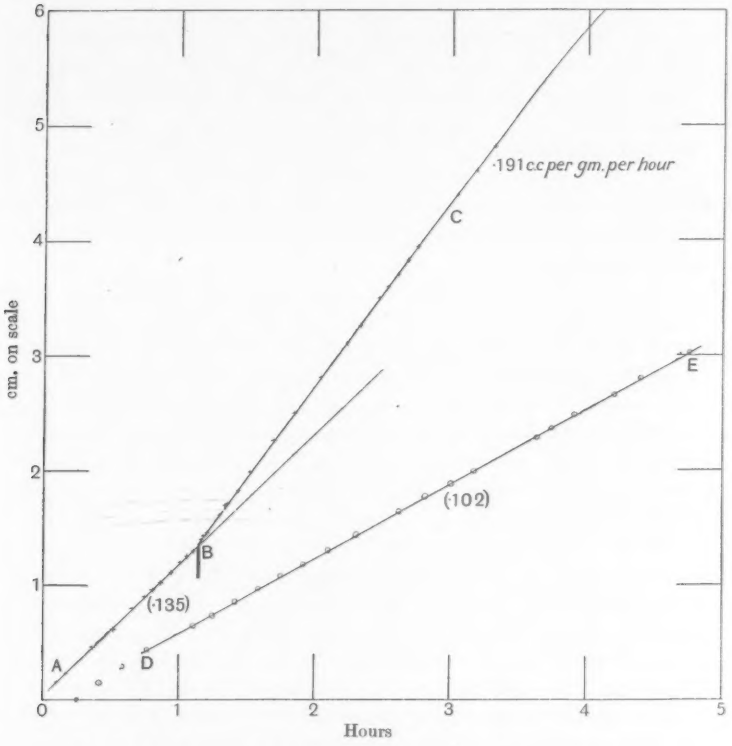

Fig. 1. The figures in brackets indicate the rate of oxygen consumption in cc. per g. per hour.

to include a small piece of bone in the preparation. These divergences were generally large in comparison with the error of reading, whereas in those experiments which show a constant rate, no divergences at all were perceptible. It would seem probable that the steady rate of respiration, remaining constant within perhaps less than 1 %, is a characteristic of a resting excised muscle, just as constant rates of metabolism appear to be characteristic of other unstimulated tissues.

Delayed oxygen consumption after stimulation.

Fig. 1, *A*, *B*, *C*, shows an experiment in which twenty single induction shocks were sent into the tissue at *B*; this was on a sartorius muscle. An immediate increase in the respiration occurred, of about 40 %, and the increased rate was maintained unaltered for about two hours after the stimulation was over, when a slow decrease commenced.

Respiration of nerve.

The experiments were done with a bundle of four or six sciatic nerves dissected out from knee to spinal cord and tied at the ends to the supporting loops of the apparatus. The nerves respired usually at a constant rate, though some slight diminution in the rate was noticed after a few hours, or after a long period of stimulation. The preliminary period of enhanced oxygen consumption, noticed with muscle, was not found with nerve; as would be expected, considering the very small effect of stimulation on the respiration of nerve, if the high initial rate is due to recovery from damage or stimulus inflicted during dissection.

The effect of stimulation was very much less than with muscle. Even tetanising currents of one minute's to half-an-hour's duration gave a very small total effect, if any, and there was practically no "delayed" consumption. Certain slight displacements of the curves of oxygen consumption were noted after long periods of stimulation, it is true, which rendered decision on the question whether the passage of the nervous impulse demands an extra supply of oxygen or not, less definite than had been hoped; but the largest displacement ever observed corresponded to an additional oxygen consumption, in excess of that which would have been used by the tissue at rest, of only 0·02 cc. per g. of tissue. This figure was obtained after fifteen minutes' continuous stimulation. Usually the effect, if any, was less than this. Whether the displacements were due to the tissue using more oxygen is very doubtful, however, since in a control experiment with moist twine in the chamber, a similar effect was actually observed. I think that the heating effect of the prolonged current passing in the chamber may be the cause of the slight displacements, though it is not easy to see exactly how this could bring about an effective increase in the volume of the chamber except by an increase of pressure so great as actually to shift the ground joints slightly.

Although the displacements were probably instrumental in origin, and therefore no positive conclusion can be drawn from them, yet the important negative conclusion appears that tetanus of fifteen minutes' duration does not cause the nerve to absorb more than 0·02 cc. per g. in excess of the resting consumption. The figures given by Tashiro [1913] for the frog's sciatic, recalculated, are for resting tissue 0·167 cc. per g. per hour, and for "stimulated," 0·43 cc. This is an additional consumption of 0·066 cc. per g. in fifteen minutes' stimulation, or three times as large as the maximum effect in my experiments, which were conducted at 22°.

Supply of energy for cell activity.

The energy for cell activities appears to be supplied ultimately by the oxidations within the cell. If however the oxygen consumption can be taken as an index of the progress of oxidation, it is clear from Fig. 1 that the supply of energy takes place, under the experimental conditions, long after the activity has ceased, an inappreciable fraction of the total energy being supplied actually during the period of contraction. That the delayed oxygen consumption is not a slow restoration of oxygen to some intracellular store (such as the "inogen") which is ready to supply large quantities of energy suddenly by oxidation at the moment of stimulation, follows from the arguments brought by Fletcher and Hopkins [1915] against the existence of any such store of oxygen within the cell; it is probable then that the oxidation occurs simultaneously (or nearly so) with the absorption of oxygen and the CO_2 evolution, and that the enhanced oxygen absorption corresponds in time with the actual oxidation or energy supplying reaction.

At the moment of contraction, the muscle fibre must work by drawing on stores of potential energy within the tissue, and it appears that the function of the oxidations is to restore the potential energy of the muscle to its normal resting level. The muscle fibre is further so constructed that the demand for replenishment of these stores of potential energy, available for future activity, is automatically supplied: for the activity of the cell leaves behind a condition leading immediately to an accelerated oxidation.

It does not seem possible to do more than conjecture the function of the steady rate of oxygen consumption in the resting tissue, nor to decide whether it is connected with the maintenance of the normal level of potential energy in the tissue or with some other activity. It may be that the resting respiration is an index of an anabolic process, compensating, and proceeding at an equal rate with, some such catabolic process as the survival formation of lactic acid, observed to occur in resting tissues at a constant rate; but direct evidence that these processes are connected appears completely lacking.

Note on the method of Shiro Tashiro [1913] *for the estimation of very small amounts of carbon dioxide.*

Miss D. M. Moyle and I, in preliminary studies made with a view to utilising the above method for estimation of the CO_2 output of nerve, met with quite unexpected difficulties, which are perhaps worth recording, as they appear to make it doubtful whether the drop of baryta solution is quite as reliable as has been supposed for *quantitative* estimations of small amounts of CO_2 in air.

The quantitative method depends on the alleged fact that a nearly hemispherical drop of baryta solution, standing at the top of a narrow column of the same liquid, in a chamber of about 15 cc. capacity, shows a visible precipitate of barium carbonate when the total CO_2 content of the chamber exceeds

1×10^{-7} g., but does not do so if the amount of CO_2 is less than this. The time required for full development of the visible precipitate is stated as ten minutes. Analyses are made by determining the volume of gas required to give the precipitate.

The first step we took was to test the accuracy of this figure, as it is fundamental to the analysis that the critical amount of CO_2 required should be accurately known as well as the limits within which it is reproducible in successive experiments; and it was clearly of importance to determine, before relying on the method, whether any modification in the design of the instrument or the conditions of use would affect the critical amount of CO_2. All measurements and manipulations of the gases were done over mercury; the apparatus consisted of (*a*) a reservoir of CO_2-free air, the preparation and storage of which according to Tashiro's instructions presented no difficulty, (*b*) measuring instruments and siphon pipettes, for transferring the gases and mixing the standard mixtures required for the calibration, (*c*) a chamber in which the drop of baryta could be formed and observed, and into which the gas mixture could be introduced.

The calibration was very disappointing. In a series of experiments the following results were obtained for (col. 2) an amount which just did give, and (col. 3) an amount which just did not give, a visible precipitate, on close examination with a good lens.

Experiment No.	Ppt. obtained	No. ppt. visible
1	$1{\cdot}13 \times 10^{-7}$	$0{\cdot}91 \times 10^{-7}$
2	0·59 ,,	0·55 ,,
3	0·97 ,,	0·83 ,,
4	1·94 ,,	1·83 ,,
5	0·75 ,,	0·71 ,,
6	0·78 ,,	0·76 ,,
7	—	1·76 ,,
8	1·46 ,,	1·27 ,,

It would appear that the drop of baryta in our hands was not more than a delicate qualitative test for CO_2 and could not be relied on for quantitative measurements.

Several variations of the technique were attempted with no better results, and the conclusion was reached not only that the method would prove extremely laborious if a long series of analyses had to be undertaken, but that we could not obtain results of even 50 % accuracy from it.

It appears not improbable that some nuclei analogous to those required for the crystallisation of supersaturated solutions may be an important factor in determining whether or no a precipitate becomes visible, and if this be the case, it is not surprising that somewhat varying amounts of CO_2 were found necessary to give the precipitate.

REFERENCES.

Adam (1920). *Biochem. J.* **14**, 679.
Fletcher (1898). *J. Physiol.* **23**, 29.
Fletcher and Hopkins (1915). *Proc. Roy. Soc.* B, **89**, 444.
Tashiro (1913). *Amer. J. Physiol.* **32**, 107 137.

XL. A CRITICAL STUDY OF THE METHODS OF ESTIMATING CHOLESTEROL AND ALLIED SUBSTANCES.

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In recent years two methods, quite different in type, have come into common use for the estimation of cholesterol and its esters: a gravimetric method based on the formation of a highly insoluble molecular compound of cholesterol with digitonin, and a colorimetric method based on the Borchard-Liebermann reaction with acetic anhydride and sulphuric acid.

Both methods give good results when pure cholesterol is in question, but difficulties arise when mixtures of different sterols, or mixtures of cholesterol and oils, as, for example, the ether extracts of tissues, faeces, etc., have to be dealt with.

Gravimetric method.

In the year 1901 Ransom [1901] made the important discovery that the haemolytic action of saponin on blood corpuscles was prevented by the addition of cholesterol, and concluded that a kind of affinity existed, or a solubility equilibrium was set up, between the saponin and cholesterol. This discovery was of far-reaching interest, and soon led to experiment with other haemolytic materials. Thus it was found that cholesterol had an inhibitory effect on haemolysis by bee poison, garden-spider poison, snake poison, etc. Further investigations by Hausmann [1905] and by Abderhalden and Le Count [1906] proved that whereas cholesterol and phytosterol inhibit the action of saponin their esters do not. In 1909 Fraser and Gardner [1909] successfully made use of these facts to compare the relative cholesterol content of the sera of rabbits in dietetic experiments.

For some time it remained doubtful whether the inhibitory action of cholesterol was due to chemical action or to some physical process such as adsorption. Arrhenius [1907] regarded it as analogous to the neutralisation of toxin by antitoxin. The question was, however, definitely settled in 1909 by Windaus [1909], who made use of digitonin in his experiments, because this saponin was crystalline, had been proved to be a single substance and its

empirical formula had been determined. He found that when digitonin dissolved in 95 % alcohol was mixed with an alcoholic solution of cholesterol a compound of digitonin with cholesterol, insoluble in alcohol, was formed. This had the formula $C_{82}H_{140}O_{29}$. He also found that similar insoluble compounds were formed with sitosterol, β-cholestanol, coprosterol and stigmasterol.

In 1910 Windaus [1910] proposed a method of estimating cholesterol and its esters, based on this observation, which has since come into general use. About the same time Fraser and Gardner [1910] published a similar method, differing somewhat in detail, for attaining the same end, for which they claimed some advantages. In 1914 Thaysen [1914] published a detailed critique of the digitonin method and emphasised the necessity of using $1\frac{1}{2}$ to $2\frac{1}{2}$ % excess of digitonin in order to obtain accurate results. This conclusion we can fully confirm. Thaysen also pointed out the conditions necessary to attain complete hydrolysis of cholesterol esters by sodium ethoxide. In general he completely confirmed the validity of the digitonin method.

Alcohols of the sterol group can be divided into two classes according as they form insoluble digitonides or not, though as yet reliable data are wanting to complete the groups.

Group I. Insoluble digitonides.

The unsaturated alcohols—cholesterol, sitosterols, and probably other phytosterols, stygmasterol.

The saturated alcohols—β-cholestanol, coprosterol.

Group II. No insoluble digitonides.

The saturated alcohols—ψ-coprosterol, ϵ-cholestanol [Windaus 1916].

The unsaturated alcohol ψ-cholesterin [Windaus and Resau 1915].

Also α-cholestanol, though this has been shown by Windaus and Uibrig [1913] to be an amyl derivative of cholesterol.

The amorphous substances which accompany coprosterol in faeces [Gardner 1921] if these really belong to the sterol group.

The esters of Group I are not precipitated by digitonin.

Cholesterol digitonide. According to Windaus this substance is easily soluble in pyridine. He gives the following figures for its solubility in alcohols:

100 cc. of methyl alcohol at 18° dissolve 0·47 g.

100 cc. of 95 % ethyl alcohol at 18° dissolve 0·014 g.

100 cc. of 95 % ethyl alcohol at 72° dissolve 0·16 g.

100 cc. of 50 % ethyl alcohol at boiling point dissolve 0·03 g.

It is insoluble in cold water, acetone, ether, ethyl acetate, and benzene; and gives the Borchard-Liebermann reaction. It dissociates into its constituents on heating in the vapour of boiling xylene, and some slight amount of dissociation also occurs on prolonged boiling with methyl alcohol.

Sitosterol digitonide is quantitatively precipitated in crystalline needles and has similar properties. The stigmasterol compound is still more insoluble.

β-Cholestanol digitonide. β-Cholestanol according to Windaus forms a slightly more soluble compound, 100 cc. of 95 % alcohol dissolving 0·21 g. We found,

by digesting β-cholestanol digitonide for several days with ethyl alcohol of 96 % by weight at temperatures ranging from 10–18° with occasional shaking, values of 0·034 to 0·067 per 100 g. of solvent. At the boiling point 0·27 g. was dissolved. Working in a similar manner the solubility in commercial ether at 10–17° was found as an average of three estimations to be 0·0084 g., and in light petroleum 0·01 g. per 100 g. of solvent.

Coprosterol digitonide we found to be much more soluble in alcohol than the digitonides of other members of the group. Thus 100 g. of 96 % alcohol (by weight) dissolved at 10°, 0·143 g.; at 15°, 0·24 g.; and at 78°, 0·83 g.

In commercial ether at 10–17° the average of three estimations was 0·0077 g., and in light petroleum 0·0025 g. per 100 g. of solvent.

In applying Windaus's procedure for the estimation of any of these sterols a correction may be introduced for the solubility in alcohol. This becomes the more desirable the smaller the quantity of digitonide to be weighed. It is always, however, necessary in the case of coprosterol. In this case also a considerable excess of digitonin should be used. In the procedure recommended by Fraser and Gardner [1910] such a correction is obviated, since the alcohol is evaporated off at a low temperature before washing the precipitated digitonide. In this case any accompanying oils should be washed away with ether or light petroleum, and the excess of digitonin by warm water. The precipitate should not be washed with alcohol.

In both methods a very slight error will be introduced by the ether or light petroleum washing, owing to the slight solubility of the compounds in these solvents, but in most cases the error is negligible. When these methods are used to estimate free sterol in a large excess of fat, or non-sterol unsaponifiable matter, it is possible that the solubility of the compounds in ether in the presence of such substances may be increased and a somewhat greater error introduced. A long experience of the digitonin method as applied to the analysis of ether extracts of faeces, tissues, milks, etc., has however convinced us that such error is not serious, provided the quantities of compound to be weighed are not too small.

As the compound weighed is about four times the weight of the sterol, accurate results are attainable with very small quantities of sterol. In order to illustrate the reliability of the method in the case of the estimation of very small quantities of β-cholestanol and coprosterol we give some results in the following table. The estimations were made by the procedure of Fraser and Gardner, the precipitate being well washed with ether, as would be the case in an actual estimation in a fat, and finally with warm water.

Sterol	Weight of sterol used in g.	Weight of digitonin compound in g.	Calculated sterol (anhydrous)
β-Cholestanol crystals	0·0108	0·0459	0·0112
,, ,,	0·0176	0·0705	0·0172
,, ,,	0·0177	0·0711	0·0174
Coprosterol ,,	0·1022	0·4190	0·1022
,, ,,	0·0109	0·0451	0·0110

Colorimetric method.

The colour methods usually employed depend either on the Salkowski reaction, as developed by Weston and Kent [1917] and others, or on the Liebermann reaction as originally proposed by Grigaut [1910, 1913].

In general a solution of cholesterol in acetic anhydride gives on addition of a few drops of strong sulphuric acid a succession of colours: red, blue, bluish green, and then a beautiful green. The latter gradually fades and in a few hours leaves a brownish solution. The spectral characters of the different stages of this reaction have been studied by Lifschütz [1913], who also showed that "oxycholesterin" presents the same succession of colours with the same spectral characters.

The Liebermann reaction is also given by coprosterol and the various phytosterols, and in a modified manner by chortosterol [Dorée and Gardner 1908], isocholesterol, and some other members of the sterol group.

This reaction is also given according to Wieland and Weil [1912] by cholatrienic acid and other bile derivatives. Many resinous substances give with acetic anhydride and sulphuric acid a reddish-brown colour, which sometimes develops on standing a green tint, or a green fluorescence. The amorphous substances which accompany coprosterol in faeces also give this reaction in a modified manner [Gardner 1921].

Obviously the presence of these substances would exercise a disturbing effect on the estimation of cholesterol by colour reactions, and various observers have been at pains to show the absence of such disturbing factors from the unsaponifiable matter of tissues. Thus Unna and Goloditz [1909] showed that in skin there is no trace of isocholesterol, or other substance capable of interfering with the reaction. Mauriac and Defaye [1912] showed that the presence of oleic acid has no influence on the reaction, but on the other hand Lifschütz [1908] by oxidation of oleic acid with permanganate obtained a substance which gave the reaction. Grigaut [1911] also was at some pains to show that the cholesterol extracted from serum was sufficiently free from disturbing substances by asserting the constancy of melting point and optical properties of his cholesterol.

Grigaut in his monograph [1913] also recognises that the reaction is a progressive one and would be affected by the mass of various reagents, and mentions the importance of always using in comparative estimations the same quantity of sulphuric acid. Autenrieth and Funk [1913] also lay stress on this point, as affecting the degree and type of colour produced. None of these authors, however, in any way elucidates the effect produced by varying the volume of the acid. With regard to the estimation of the other members of the sterol family, the only mention, as far as we are aware, is that made by Myers and Wardell [1915], who attempted to estimate coprosterol in faeces colorimetrically. They said that coprosterol had a value about one-third that of cholesterol.

The present investigation was, therefore, undertaken with a view to determining whether other members of the sterol group gave the colour reactions in such a way as to be comparable with cholesterol, and how variations of conditions affect the ultimate colour produced.

Experimental.

The production of colour in solutions of cholesterol and coprosterol was first compared.

The conditions used were those recommended by Autenrieth and Funk, *i.e.* 10 cc. of a chloroform solution of the sterol, of the order of $\frac{2}{10000}$ molecular, were treated with 4 cc. of acetic anhydride and 0·2 cc. of conc. sulphuric acid. The colour was developed in the light and at room temperature.

Under these conditions the colour developed slowly in the case of cholesterol, the first trace of colour being green. With coprosterol the development of the colour was still slower, and after half an hour the solution was only a very pale green. The deepening of the tint during $1\frac{1}{2}$ hours was still slight, and the colour was never comparable with that of the equivalent cholesterol solution, but on the addition of more acid a marked increase in depth of colour became apparent.

A detailed study of the effect of acid was therefore undertaken. The results are summarised in the following table:

Volume of sulphuric acid cc.	10 cc. chloroform solution of cholesterol (0·007 g. per 100 cc.) +4 cc. acetic anhydride	10 cc. chloroform solution of coprosterol (0·007 g. per 100 cc.) +4 cc. acetic anhydride
0·2	Green colour slowly developed. Maximum depth in 15–20 minutes	Very faint green colour, gradually developing during 2–3 hours
0·4	Blue developed first, and in about 5 minutes the colour was pronounced blue green. No apparent deepening on further standing	Colour began to appear in about 8 minutes, and developed rather more rapidly than above. No further deepening after 1 hour. Final shade not so deep as the cholesterol
0·5	Mauve colour first, changing rapidly to blue, then green. Full development of colour in about 5 minutes	Colour developed more quickly than above. Blue tint first seen, slowly changing to green. Colour in $\frac{3}{4}$ hour was identical with that of the cholesterol, but the latter had by then faded to some extent
0·6	Ditto	Ditto
1·0	Pink → mauve almost at once, then violet, blue, blue green and green. The whole series of changes occupied less than 2 minutes, and full depth of colour appeared to have been produced	Colour appeared in $\frac{1}{2}$ minute—slate-blue first, deepening rapidly, though less quickly than cholesterol. In 5 minutes the colour was slightly paler than the cholesterol, and rather more bluish green in quality
1·5	Changes as above	Pink → mauve colour almost instantly → blue → green. Colour deepened rapidly and rate and depth were about identical with cholesterol
2·0	Changes instantaneous, but sulphuric separated as an oil from solution	As cholesterol

In these reactions the depth of colour was compared by eye only in Nessler glasses, as qualitative results were first required. The reaction was however

clearly a time reaction, the rate depending on the amount of acid. Luden [1917] attempted unsuccessfully to show this sequence of colours by using smaller quantities of reagents, but reached some sign of them in very concentrated cholesterol solutions, though the colours were apparently less distinct than those noted above.

In order to determine whether the addition of the larger quantities of acid has any effect on the *final* depth of colour, readings were taken in a Dubosc colorimeter, using the same standard cholesterol solution—0·0394 in 250 cc. chloroform—in each case.

Two difficulties were encountered: firstly, the colours with greater quantities of sulphuric acid were much more blue-green than the tint with 0·2 cc. sulphuric acid, and secondly the colours were less transparent and therefore more difficult to compare. Much reliance, therefore, could not be placed on the results, but with the standard (containing 0·2 cc. of acid) set at 15 in the scale, the mean of a somewhat irregular series of readings for the solution containing 1·2 cc. acid was 15·1. It appears probable that the final depths of colour in the two cases are not divergent, but the practical difficulties point to the necessity of using the same volume of sulphuric acid throughout any series of estimations.

The influence of temperature was next investigated. The series of reactions quoted above were performed at room temperature, *i.e.* about 10–10·5°. Considerable divergence of opinion, however, seems to be prevalent as to what is the best temperature for the reaction. Luden [1917] uses room temperature, presumably because of the short period of colour induction allowed in her experiments before beginning colorimetric estimation. Autenrieth and Funk [1913] recommend a temperature of 30–35°, as also does Czonka [1916]. Bloor [1916], on the other hand, prefers room temperature. To test the effect of varying temperature duplicate experiments at room temperature (10°) and at 35° were made. In each case 10 cc. of the above chloroform solution and 4 cc. of acetic anhydride were treated with the selected quantity of acid, *e.g.* 0·2 cc., and allowed to stand 15 minutes. In every case, with the smaller amounts of acid, the higher temperature caused the production of a brilliant yellow green colour, as compared with an emerald green at the lower temperature. This made comparison in the colorimeter harder, but the scale readings at the higher temperature were systematically somewhat lower than at room temperature, for instance, using 0·2 cc. sulphuric acid with the scale at room temperature set at 15, the mean of four readings of that of the higher temperature solution was 14, so that a slightly greater depth of colour had evidently been produced. With 1·2 cc. of acid at the higher temperature the colour was again less transparent, but the shade bluer, which made comparison easier.

From our results it is evident that in any series of estimations it is necessary to use sensibly the same temperature throughout.

Experiments with varying quantities of acetic anhydride showed that this factor was unimportant, provided the substance was present in large excess.

It was also found best to use concentrated sulphuric acid, since traces of water, as was also observed by Grigaut, retard the action. To test the accuracy of the estimations under optimum conditions a large number of experiments were made. At first we used a dilution method. The colour was developed in the solutions to be compared and the darker was then diluted with a mixture of chloroform, acetic anhydride and sulphuric acid of the same composition as the fluid to be diluted, until equality of colour with the paler solution was attained. Matching was done with the naked eye. The method proved unsuitable for the following reasons:

(1) Matching by eye was not sufficiently delicate.

(2) On dilution the shade of green often became more yellow in quality.

(3) The diluted solution on standing was often found to darken appreciably in colour, so that the end point was often overshot, *i.e.* the time factor came in.

(4) The method was not very sensitive. The addition of 1 cc. or more of the solution appeared to make only a very slight variation in the depth of colour.

Accurate results were obtained only when the solutions to be compared did not differ very much in strength. With solutions of molecular concentration above $\frac{4}{10000}$, which required considerable dilution, grave errors crept in, and the results as a rule were much too high.

This method was therefore discarded and a colorimeter used. A series of known cholesterol solutions were estimated against a standard solution. In each case 10 cc. of chloroform solution plus 4 cc. of acetic anhydride were treated with 1·2 cc. sulphuric acid—*i.e.* the maximum quantity that could safely be added without risk of separation of the acid, and the temperature was 35°. The following figures are typical of the results obtained.

Cholesterol solution taken (g. per cc.)	Strength found (g. per cc.)
0·00371	0·00378
0·00329	0·00310
0·00288	0·00280

Coprosterol. Coprosterol solutions were also investigated quantitatively. 10 cc. of a standard coprosterol solution (0·0658 g. per 100 cc. $CHCl_3$) and 4 cc. acetic anhydride were used in every case. The following example will suffice. To *A* 0·2 cc. sulphuric acid was added and to *B* 1·2 cc., both at room temperature, and to *C* 0·2 cc. at 35°. At the end of 15 minutes *B* was obviously darker to the eye than *A* or *C*. The solutions were allowed to stand for another 10 minutes and then compared in the colorimeter. With *A* set at 15 on the scale, the reading of *B* was 3·3, and of *C* 8·3. Obviously therefore both volume of acid and temperature are of vital importance.

Estimations of coprosterol against standard solutions of cholesterol, using a Dubosc colorimeter, lead to the same conclusion.

Using a cholesterol solution containing 0·000412 g. per cc. and a coprosterol solution of 0·000658 g. per cc., 0·2 cc. sulphuric acid was added to a

mixture of 10 cc. of each with 4 cc. acetic anhydride, and they were allowed to stand at room temperature for 30 minutes and then compared.

With the cholesterol scale at 5, the mean reading of the coprosterol solution was 10·6, indicating 0·00019 g. per cc. instead of 0·000658.

On repeating the experiment at 35°, with the cholesterol set at 15, the mean coprosterol reading was 13·25, equivalent to 0·000466 g. per cc. instead of 0·000658.

Using, however, 1·2 cc. of sulphuric acid at room temperature a value of 0·000636 was obtained, and at 35° 0·00068. The higher temperature had the advantage of favouring the production of the same quality of colour in each solution.

The time of colour induction, as already indicated, is much longer in the case of coprosterol than in the case of cholesterol. On comparing solutions in the above proportions using 0·2 cc. of acid the colours approximate at the end of about 2·5 hours, but during this period the colour of the cholesterol solution has appreciably faded. We found that in the case of a cholesterol solution, using 0·2 cc. of acid, fading began to be appreciable at the end of half an hour. Consequently in comparisons of cholesterol with coprosterol or mixtures of cholesterol and coprosterol sufficient sulphuric acid should be added to get the maximum colour development in each as rapidly as possible, certainly within 30 minutes. If these conditions are fulfilled accurate estimations can be made.

ψ-Coprosterol. The behaviour of this substance was investigated in a similar manner, and compared with that of coprosterol. With solutions of approximately $\frac{2}{10000}$ molecular concentration the colour of the ψ-coprosterol solution was insufficient to make comparison possible. Solutions of ψ-coprosterol of $\frac{4}{10000}$ and $\frac{6}{10000}$ molecular concentration were therefore used.

With 0·4 cc. of sulphuric acid (to 10 cc. chloroform sol. + 4 cc. acetic anhydride) no immediate coloration appeared, but after about 25 minutes a faint yellow green was seen. With 0·8 cc. acid the colour was obvious in 5–10 minutes, and was definitely pale green in 25 minutes. Even with 1·2 cc. of acid the colour did not appear immediately. No sign of mauve was noted, but a dirty grey colour came first. After about 5 minutes the green colour developed slowly. Increased temperature was found to speed up the reaction, so that with sufficient acid colour appeared almost at once, but the final shade was never really comparable in quality with that of cholesterol or coprosterol, and certainly not in depth.

A series of readings were however taken, as nearly as we could manage, using optimum conditions with the following results:

ψ-coprosterol	weighed	0·017 g.	found 0·0068 g.
,,	,,	0·023 ,,	,, 0·0053 ,,
,,	,,	0·023 ,,	,, 0·0062 ,,

Evidently ψ-coprosterol cannot be estimated colorimetrically against either

cholesterol or coprosterol, and if present in a mixture with either of these substances would vitiate any colour estimations of the latter.

β-Cholestanol. According to Willstätter and Mayer [1908], when a chloroform solution of β-cholestanol was treated with acetic anhydride and two drops of sulphuric acid, it remained colourless, but after adding double the mass of reagents the solution, without progressive colour changes, became pale green and showed fluorescence.

A specimen of β-cholestanol prepared by Ellis and Gardner [1918] by reducing until further change in optical rotation no longer took place, gave very faint colour changes with plenty of acid—first blue and then green—but the depth of colour was so feeble as to lead to the conclusion that probably a specimen absolutely free from cholesterol would give no colour.

Esters. The reactions of cholesteryl acetate, benzoate and stearate were examined, and also those of coprosteryl chloroacetate.

The colour reactions in the first three substances were given as easily as with cholesterol itself. The colour developed with either 0·2 cc. acid or 1·2 cc., both in range and final shade, was identical with the cholesterol standard.

With coprosteryl chloroacetate, on the other hand, the colour changes were very slow. The first shade to appear was yellow, changing slowly to a dirty grey-green, and at least 10 minutes elapsed before the normal green shade was apparent. The depth of colour never approximated to that given by a coprosterol or cholesterol solution of equivalent strength. In the case of the cholesteryl esters the quantitative results were invariably rather on the high side. For example, cholesteryl benzoate and acetate tested against cholesterol gave:

Weight benzoate taken 0·001417 g. found 0·001483 g.
„ acetate „ 0·001656 „ „ 0·00184 „

In the case of stearate the figures were generally slightly higher than truth.

Amorphous sterols from faeces [*Gardner* 1921].

These substances in chloroform solution with acetic anhydride and sulphuric acid give a brown colour, rather like ferric acetate in shade. This gradually changes through a dusky green to a brighter green. The rate of change depends on the quantity of sulphuric acid used and the temperature. The final green colour is however quite different in quality from that of cholesterol or coprosterol, being a yellowish green rather than a bluish green. Consequently it is very difficult to compare the depth of colour in a colorimeter with a standard coprosterol colour, and the results are of little value. Nevertheless we made comparisons as well as we could against both coprosterol and ψ-coprosterol, using a maximum quantity of sulphuric acid, and room temperature. The time factor was more or less eliminated by taking readings first at the end of 30 minutes and again at the end of 60 minutes, but this proved unnecessary as the readings were identical.

Assuming for purposes of calculation that the amorphous sterols had the formula of coprosterol the following results were obtained, using different specimens against a coprosterol standard:

	Composition of sample		Amount weighed in g.	Calculated amounts
(1)	83·75 C	12·01 H	0·0156	0·0087
(2)	84·16 „	12·2 „	0·008	0·004
(3)	83·45 „	12·09 „	0·0075	0·0032
(4)	83·70 „	12·36 „	0·04	0·025
(5)	84·04 „	12·23 „	0·0235	0·012

It will be seen that the calculated quantities are about half the actual. Using a ψ-coprosterol standard the following figures were obtained:

	Amount weighed g.	Calculated g.
(1)	0·056	0·132
(2)	0·0215	0·032
(3)	0·044	0·071

From these figures the depth of colour produced under optimum conditions by these substances would appear to be about half-way between that of coprosterol and that of ψ-coprosterol, but more nearly allied to the latter in quality. Obviously the presence of such substances in any extract would render the accurate colorimetric estimation of cholesterol and coprosterol in such extract quite impossible.

Examination of the ether extract of faeces.

In the application of these methods to the analysis of the unsaponifiable matter of faeces, which contains coprosterol, β-cholestanol, cholesterol, possibly phytosterols from vegetable food, amorphous sterols and alcohols, such as cetyl alcohol, etc., the digitonin method permits of the quantitative separation of the *total* coprosterol, β-cholestanol, cholesterol and phytosterol from the amorphous sterols and non-sterol alcohols. The precipitated sterols may be extracted from the digitonides by heating in the vapour of boiling xylene as recommended by Windaus. *There is no simple method, however, by which small quantities of these recovered sterols can be analysed.* If sufficient quantity of the material were available the cholesterol (and perhaps any phytosterol) could be approximately separated from the coprosterol and β-cholestanol by precipitating the cholesterol dibromide from an ethereal solution of the sterols by means of a suitable solution of bromine in glacial acetic acid at a low temperature. The cholesterol may be recovered from the dibromide by reduction with zinc dust and glacial acetic acid. The coprosterol may also be separated, after the bulk has been isolated by fractional crystallisation, from β-cholestanol by conversion into ψ-coprosterol and precipitation of the β-cholestanol by digitonin, according to the plan adopted by Windaus [1916] in his work on the separation of the three dihydrocholesterols obtained by the reduction of cholesterol by hydrogen in the presence of nickel. A rough approximation of the amount of β-cholestanol in the mixture could also be

obtained by the colorimetric method, as β-cholestanol either gives no colours or very faint ones.

Colour methods as applied directly to the unsaponifiable matter of faeces are quite useless, as it is impossible to make any accurate comparison of the colour obtained with the unsaponifiable matter with that of a coprosterol standard either by naked eye comparison in reagent glasses or by a colorimeter owing to the quite different quality of the green shades obtained. Even if this could be got over it would be difficult to assign any meaning to the results, since, though cholesterol and coprosterol give comparable colour reactions, β-cholestanol gives only feeble colours, if any, and the amorphous sterols give not only a yellower green but a colour of a lower order of intensity. This will be readily seen from the following estimations by the two methods of the unsaponifiable matter of the faeces of a number of individuals fed on practically the same diet.

	Sterols by digitonin, reckoned as coprosterol per g. of unsaponifiable matter	Sterols by colour method, reckoned as coprosterol per g. of unsaponifiable matter
(1)	0·511	0·513
(2)	0·575	0·431
(3)	0·606	0·382
(4)	0·632	0·418
(5)	0·696	0·434
(6)	0·629	0·446*
(7)	0·472	0·563
(8)	0·671	0·709*
(9)	0·594	0·678*
(10)	0·517	0·852*

* These samples were especially difficult to compare owing to the marked yellow shade.

The ether extracts of tissues, etc.

With the ether extracts of tissues, animal fluids, such as milk, serum, etc., according to our own long experience and that of numerous other workers, the digitonin method gives accurate results, and has the important advantage that both cholesterol and its esters can be estimated, though where the esters are relatively small in quantity experimental errors are correspondingly larger. It must not be forgotten however that though the quantity of cholesterol in fats may be accurately estimated, the accuracy of the results when expressed as a percentage of the original tissue naturally depends on the assumption of the efficient extraction of the fatty matter from the tissue, but this is a question outside the present inquiry.

The colorimetric methods give satisfactory results on the whole with serum or blood, but with other tissue extracts the estimations are less trustworthy, owing to greater or less differences in quality between the shades obtained with such tissues and the cholesterol standard used, which render the comparison of the colour intensities less precise. When the cholesterol is completely precipitated from the unsaponifiable matter of the fat of a tissue,

such as muscle or liver, or of milk, by digitonin, the unprecipitated portion of the unsaponifiable matter in chloroform solution always gives with acetic anhydride and sulphuric acid a marked brownish-red coloration, which often changes in a few minutes through a dusky green to a yellowish green, and even a bright grass green. Also, when ordinary alcohol is boiled with alkalis, diluted and extracted with ether, some substance is extracted by the ether which in chloroform solution gives with acetic anhydride and sulphuric acid a brown colour, which often becomes more or less green [*vide* Gardner and Fox, 1921]. Whether the green colour observed with the cholesterol-free unsaponifiable matter is due to the inherent properties of this substance, or to traces of cholesterol digitonide remaining in solution, or to the outside cause referred to has not yet been decided. Nevertheless the production of this green introduces an error into the colorimetric results, and in any case the brown colour always obtained modifies the quality of the shade of colour due to the cholesterol. The digitonin method necessitates the use of a certain minimum quantity of material to give an amount of digitonide sufficient for reliable weighing; in the case of blood, for instance, this means at least 30 to 50 cc. The colorimetric method on the other hand is of the nature of a micromethod, and in the case of blood can be carried out on 2 cc. or even less. This, of course, is a very great advantage in the study of the living animal and the human subject. Very fair agreement between the digitonin and colour methods has been recorded by Klinkert in the case of serum [1912, 1913]. In the case of blood, milk, etc., accurate comparative results can, we believe, be obtained in a series of experiments provided *rigidly* comparable conditions are adopted. The results should also at some stage be checked by the digitonin method. Unless these precautions are observed serious errors may arise.

REFERENCES.

Abderhalden and Le Count (1906). *Zeitsch. exp. Path. u. Ther.* **2**, 199.
Arrhenius (1907). Immunochemistry, 8°. New York.
Autenrieth and Funk (1913). *Münch. med. Woch.* **60**, 1243.
Bloor (1916). *J. Biol. Chem.* **24**, 227.
Czonka (1916). *J. Biol. Chem.* **24**, 431.
Dorée and Gardner (1908). *Proc. Roy. Soc.* B, **80**, 213.
Ellis and Gardner (1918). *Biochem. J.* **12**, 72.
Fraser and Gardner (1909). *Proc. Roy. Soc.* B, **81**, 230.
—— (1910). *Proc. Roy. Soc.* B, **82**, 559.
Gardner (1921). *Biochem. J.* **15**, 244.
Gardner and Fox (1921). *Biochem. J.* **15**, 376.
Grigaut (1910). *Soc. Biol.* 791, 827.
—— (1911). *Soc. Biol.* Nov. 11, 513.
—— (1913). Le cycle de la cholesterinémie, Steinheil. Paris.
Hausmann (1905). *Beiträge* **6**, 567.
Klinkert (1912). *Med. Tijdsch. von Geneesk. Amst.* 720.
—— (1913). *Berl. klin. Woch.* **1**, 820.
Lifschütz (1908). *Zeitsch. physiol. Chem.* **56**, 441.
—— (1913). *Biochem. Zeitsch.* **48**, 373; **5** , 212; **62**, 219.

Luden (1917). *J. Biol. Chem.* **24**, 463.
Mauriac and Defaye (1912). *Soc. Biol.* No. 26.
Myers and Wardell (1915). *J. Biol. Chem.* **36**, 147.
Ransom (1901). *Deut. med. Woch.* **27**, 194.
Thaysen (1914). *Biochem. Zeitsch.* **62**, 89.
Unna and Goloditz (1909). *Biochem. Zeitsch.* **20**, 469.
Weston and Kent (1917). *J. Biol. Chem.* **28**, 383.
Wieland and Weil (1912). *Zeitsch. physiol. Chem.* **80**, 287.
Willstätter and Mayer (1908). *Ber.* **41**, 2199.
Windaus (1909). *Ber.* **42**, 238.
—— (1910). *Zeitsch. physiol. Chem.* **65**, 110.
—— (1916). *Ber.* **49**, 1724.
Windaus and Resau (1915). *Ber.* **48**, 851
Windaus and Uibrig (1913). *Ber.* **46**, 2487.

XLI. NOTE ON A SOURCE OF ERROR IN THE COLORIMETRIC METHODS FOR THE ESTIMATION OF CHOLESTEROL IN TISSUE FATS.

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(*Received April 18th, 1921.*)

In the course of an investigation of the effect of heating the amorphous sterols of human faeces in sealed tubes with alcoholic potash, it was noticed that alcoholic potash which had been heated alone in a blank experiment, when diluted with water and extracted with ether, yielded to the ether a small quantity of resinous matter, which on treatment in chloroform solution with acetic anhydride and a few drops of sulphuric acid gave a marked brown colour which on standing developed a yellowish-green tint. A standard alcoholic potash solution, that had stood in the laboratory for several years and was brown in colour, after extraction and treatment in a similar manner gave a brownish-red colour which changed in a few minutes to a marked green. Another solution which was many years old and almost black in colour gave a brownish-red colour which on standing developed a slight green tinge. The same solution, however, boiled under a reflex for two hours before extraction, gave a deep brown colour, but no green tint developed. On heating 5 grams of potash with 25 cc. of methylated spirit in a closed tube at 110° for several hours a dark brown solution was obtained. This after dilution and extraction with ether yielded a brown resin, which in chloroform solution gave with the above reagents a deep reddish-brown colour, and on standing developed a strong green fluorescence. It was red by transmitted light. A specimen of aldehyde resin made by the action of aqueous soda on acetaldehyde gave a cherry-red colour, but no green. Since in estimations of the sterols of tissues by colorimetric methods the fats are hydrolysed by boiling with alcoholic potash or soda or sodium ethoxide, and in some methods of extracting fatty matter the whole tissue is destroyed by alcoholic soda, it seemed important to submit the above observation to further investigation.

The alcohol used was the duty-free non-methylated alcohol of commerce of good quality as usually supplied to laboratories. In one series of experiments measured volumes of alcohol were heated with weighed quantities of powdered caustic soda or potash in sealed tubes at 110° for 2 to 4 hours. In

another series the alkali and alcohol were heated for several hours on a water-bath in a long-necked matrass, and in a third series weighed quantities of sodium were dissolved in measured volumes of alcohol and the solutions allowed to stand some time at the laboratory temperature, or in some cases were warmed on the water-bath. The experiments were done in glass vessels and no corks or other substances which might yield soluble matter to the alcohol were used. In most cases very pale yellow solutions were obtained. These were diluted with water and extracted with ether. The ethereal solutions were then thoroughly washed by shaking with water and the ether evaporated. The pale yellow residues, which rarely exceeded a few milligrams per cent. of alcohol, were then dissolved in 5 cc. chloroform and treated with 2 cc. of acetic anhydride and 6 drops (0·4–0·5 cc.) of sulphuric acid. In every case a marked reddish-brown colour was produced, the depth depending on the amount of alcohol used. When a less quantity of acetic anhydride was used a brown precipitate sometimes separated, this however readily dissolved on adding a little more acetic anhydride. The brown solutions on standing sometimes remained unchanged, but in most cases after a few minutes' standing developed a distinct greenish tinge. In a few cases the brown colour became definitely a dull grass green. The green tinge faded on standing overnight, but the brown colour was more permanent. The minute residues left on evaporating quantities of alcohol of the order of those used in these experiments gave no appreciable reaction.

It seemed likely that these effects were due to traces of aldehydes and other similar impurities in the alcohol, and in order to ascertain whether these could be conveniently got rid of by boiling with alkali, 1 litre of alcohol was boiled in a matrass with 200 grams of caustic soda for 4 hours. The alcohol was then distilled off.

50 cc. of this distillate were heated in a sealed tube with 2 grams of caustic soda for 4 hours at 110°. The solution became pale yellow. After extraction with ether a trace of residue was obtained. This was dissolved in 5 cc. chloroform and treated with 2 cc. acetic anhydride and 6 drops of sulphuric acid. The liquid became pale brown and on standing for a few minutes changed to a greenish brown. The colour was quite different from the blue-green colour given by cholesterol, but as far as we could judge the body of colour was about the same as that given by a standard cholesterol solution containing 0·0009 gram sterol.

In another experiment 50 cc. of the distillate were boiled with 2 grams of caustic soda on the water-bath for 4–5 hours. The solution was bright yellow. On extraction 1·4 mg. of residue was obtained which was tested as above. A dusky brown solution was obtained, but it did not turn green. A specimen of alcohol which had been dried by boiling for many hours with a large quantity of quicklime, and then distilled, but which had stood in the bottle for some weeks, was also tested. 50 cc. were heated in a sealed tube with 2 grams caustic soda at 110° for 4 hours. A yellowish solution was left. This on

extraction yielded a slight residue, which on testing gave a well-marked brown colour which gradually changed to a definite dull grass green. On comparing as well as we could with a standard cholesterol we concluded that the colour was about equivalent to 1·3 mg. of cholesterol.

A control experiment by evaporating 50 cc. to dryness gave no reaction and similarly in the case of the ether used. Whether these colour reactions are entirely due to impurities, or whether they are given by chemically pure alcohol has not been investigated.

It is obvious, however, that the production of any green colour due to alcohol used even though of a different shade would introduce an error into the colorimetric estimation of cholesterol. But even when no green tint is produced the brown colour so modifies the quality of the colour due to the cholesterol as to make it very difficult to compare with that of the standard cholesterol solution with any degree of precision. This effect can be readily demonstrated by treating a chloroform solution of pure cholesterol of suitable strength with acetic anhydride and sulphuric acid and when the green stage has been reached dividing into two equal parts. If one is then diluted with an equal volume of one of the above mentioned brown solutions with an approximately equivalent body of colour, and the other with a mixture of chloroform and acetic anhydride of similar concentration the difference can be readily seen. The one is a yellowish or grass green and the other a blue green.

XLII. THE SPECIFIC ELECTRICAL RESISTANCE OF FROG'S MUSCLE.

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From the Physiological Laboratories, Cambridge and Manchester.

(*Received April 30th, 1921.*)

In a large number of experiments carried out at various temperatures during the past two years we have found incidentally the electrical resistance of a frog's sartorius muscle after death to a single phase alternating current of about 90 periods per sec., and of a strength of the order of 1 milliampere. The results are so numerous and, when expressed in terms of the specific resistance of muscle, relatively so consistent, that we thought it advisable to collect them together and to put them on record. The observations were made during the "calibration" of a muscle, killed by chloroform vapour and resting in air or oxygen on a thermopile, by the method described in our recent paper [1920]. It should be noted that the resistances are to a relatively high current and that, in the case of a muscle, Ohm's law is not exactly obeyed: indeed we have found that a 50 % increase in the current leads to about a 10 % decrease in the resistance: consequently the resistance to a different current will be different. The cause of this failure of Ohm's law is presumably some hindrance to free diffusion, either at the electrodes (platinum wires in very good contact with the muscle) or more probably at membranes in the muscle substance. The quantity observed was the resistance of a pair of sartorius muscles in parallel between two electrodes at distance l (about $2\frac{1}{2}$ cm.) apart. It is clear that the resistance of a given mass m of a muscle of uniform cross-section A is directly proportional to its length l and inversely proportional to A. But $m = slA$ where s is the specific gravity of muscle, so that putting $A = m/sl$ the resistance is directly proportional to l and also to sl/m, *i.e.* altogether to sl^2/m. Thus we may put

$$R = ksl^2/m$$

where k is the specific resistance of muscle. Thus to find k we use the formula

$$k = Rm/sl^2.$$

The value of s was found directly by weighing a frog's muscle suspended in

[1] Working on behalf of the Medical Research Council.

0·7 % NaCl solution, and had the value 1·057, say 1·06, at 10°, and with this value of s the value of k is calculated from the results of each experiment for the following table.

Table I. *Values of the specific resistance* $k = Rm/sl^2$*: α is the value of the current used, in milliamps.:* s *is put equal to* 1·06.

0°			5°			10°			15°			20°		
Mth	k	α	Mth	k	α	Mth	k	α	Mth	k	α	Mth	k	α
May	278	0·8	May	212	1·1	May	220	1·1	May	168	1·1	May	141	1·4
July	275	1·0	May	242	0·9	July	217	1·2	May	194	1·4	May	138	1·4
Oct.	293	1·0	May	214	0·8	July	184	1·1	May	179	1·3	May	164	1·2
Oct.	265	1·2	July	212	1·0	Aug.	217	0·9	June	190	0·8	July	143	1·6
Dec.	307	1·1	July	206	0·8	Nov.	192	1·4	July	158	1·4	July	148	1·5
Jan.	261	0·9	Oct.	209	1·1	Nov.	221	1·3	July	163	1·4			
Jan.	299	0·9	Dec.	230	1·1	Nov.	217	1·7	July	166	1·1	Mean	147	1·4
Feb.	320	1·1	Nov.	258	1·2				July	160	1·2			
Feb.	292	1·1				Mean	210	1·25	July	179	1·3			
Feb.	300	0·9	Mean	233	1·0				Nov.	183	2·0			
Feb.	317	0·8							Nov.	189	1·8			
Feb.	269	1·0							Nov.	162	1·8			
									Dec.	179	1·5			
Mean	290	1·0							Mean	175	1·4			

In the experiments recorded here it happened in general that the currents used at the higher temperature were greater than those used at the lower—owing to the greater resistance in the latter case. In the neighbourhood of 1 milliampere a 50 % increase in the current makes about 10 % decrease in the resistance, so that it is advisable to apply a small correction to reduce the resistances to a uniform basis of 1 milliampere. Corrected in this way the mean values given above become: 0°, 290; 5°, 223; 10°, 220; 15°, 189; 20°, 159. It is usual to employ conductivities, rather than resistances, in dealing with solutions, and the corresponding specific conductivities are: 0°, 344×10^{-5}; 5°, 448×10^{-5}; 10°, 454×10^{-5}; 15°, 529×10^{-5}; 20°, 628×10^{-5}. These quantities are plotted as circles in the figure, and the relation between conductivity and temperature is given by the solid curve (calculated as described below).

In order to compare the muscle with a solution of NaCl the broken curve in the figure is plotted to show the relation between the conductivity (at 18°) and the strength of the solution. The data were obtained from Landolt and Börnstein's tables. At 18° the specific conductivity of the muscle is 580×10^{-5}, which corresponds to that of a NaCl solution of strength 0·36 %. The muscle is in osmotic equilibrium with 0·7 % NaCl, and it is striking that the specific conductivity of the muscle is only half that of the NaCl solution with which it is in osmotic equilibrium. This lowering of the conductivity would seem to be due to the hindrance afforded to the passage of a current by the membranes and surfaces of the tissue.

The effect of temperature on the conductivity of a muscle is similar to its

effect on the conductivity of a NaCl solution. In Landolt and Börnstein's tables the conductivity of a 0·29 % NaCl solution is expressed by the formula:

$$K_t = K_{18}[1 + c\,(t - 18) + c'\,(t - 18)^2]$$

where K_t is the specific conductivity at $t°$ C., and c and c' are constants having the values, $c = 224 \times 10^{-4}$, $c' = 79 \times 10^{-6}$; the constants c and c' change only slightly with concentration, so the above formula may be taken as applying

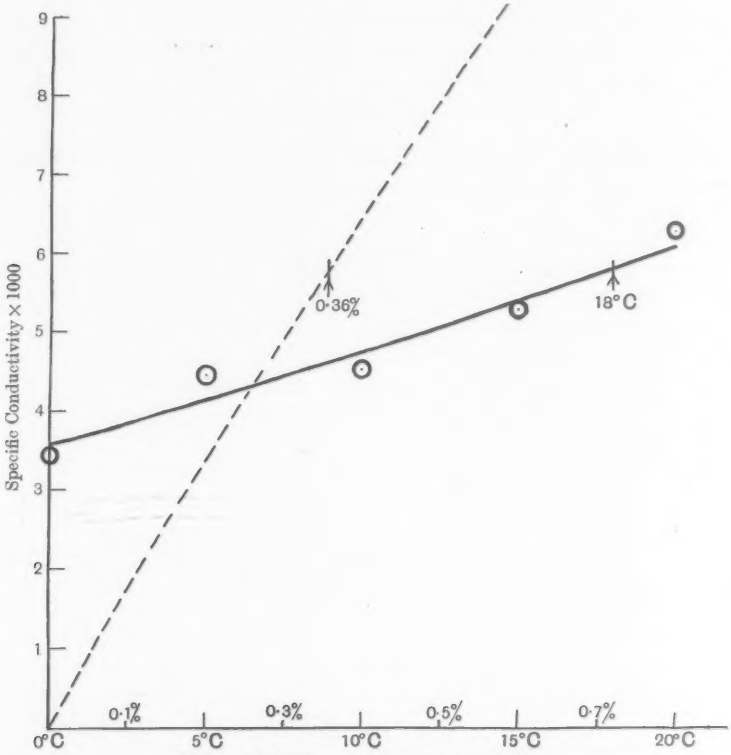

Fig. 1. (Solid Curve.) Temperature of Muscle.
(Broken Curve.) Strength of NaCl solution at 18° C.

equally to a 0·36 % solution. Putting $K_{18} = 580 \times 10^{-5}$, the value of K_t can be calculated from the above formula. In the following table the calculated values K of the specific conductivity of 0·36 % NaCl solution are compared with the observed values K' of the conductivity of the frog's muscle.

Temperature ° C.	0	5	10	15	20
$K \times 10^5$	360	419	478	542	606
$K' \times 10^5$	344	448	454	529	628

In the figure the solid curve is drawn from the calculated values K in this table. It is seen that the agreement is good, that the effect of temperature is the same on the muscle and on the salt solution, and that *at all temperatures the specific conductivity of the muscle is the same as that of a* 0·36 % NaCl *solution*. If the electrical resistance of the muscle were made up of two distinct parts, (1) the resistance of a 0·7 % NaCl solution and (2) the *separate* resistance of colloidal structures or membranes, it might be expected that the conductivity of the muscle would be influenced by temperature in a manner different from that of a salt solution. The fact that this is not so suggests that the conductivity of a muscle is lower than that of the corresponding salt solution simply because the membranes and structures of the muscle hinder the diffusion of the ions of NaCl under a potential gradient.

The experiments described here were made largely at the expense of a grant from the Royal Society.

REFERENCE.

Hartree and Hill (1920). *J. Physiol.* **54**, 84.

XLIII. ON THE STABILITY OF TRYPTOPHAN IN BARYTA HYDROLYSIS.

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Report to the Medical Research Committee.

(*Received April 22nd, 1921.*)

THE observations detailed in the following papers arose out of an attempt to estimate tryptophan, using baryta hydrolysis to set it free from the protein molecule. The method had to be abandoned, but certain observations were made on the stability of tryptophan, which seem worthy of record. The use of baryta hydrolysis for the estimation of tryptophan was first suggested by S. W. Cole, and I am greatly indebted to him for much valuable advice and assistance throughout the work, and in preparing the manuscripts.

It is well known that tryptophan is rapidly destroyed during the acid hydrolysis of proteins, so that if it is to be estimated in the free state, it must be liberated from the protein molecule, either by enzymic digestion or alkaline hydrolysis. Although digestion may prove satisfactory in the case of some proteins, von Fürth and Lieben [1920] have abundantly shown how difficult it is to set tryptophan completely free, even by prolonged digestion, about 60 % of it remaining in a peptide form. In any case, insoluble proteins like keratin could never be estimated in this way.

There is not much evidence available as to the stability of tryptophan in alkalies. Herzfeld [1913], estimating the indole produced by the action of various alkalies on proteins and tryptophan at blood heat, found that all alkalies could decompose tryptophan, sodium hydroxide being the most effective. Boiling with baryta, however, only produced a trace of indole (less than 0·5 mg.) from 1 g. of caseinogen. Miss Homer [1915], in an attempt to estimate tryptophan by bromine titration, showed that there is no apparent loss of tryptophan when caseinogen is hydrolysed with baryta for 40, 60, 80 and 120 hours respectively. Moreover, not more than 5 mg. of indole and no indole derivatives were produced during hydrolysis of 50 g. caseinogen. These conclusions are criticised by von Fürth and Lieben [1920], who argue that the figures for the tryptophan were so very low that considerable initial loss may have taken place. Further, they believe that there is an appreciable loss of tryptophan after six hours' heating with baryta on the water-bath. These statements depend on the accuracy of the formaldehyde colour reaction as a method of estimating tryptophan in unhydrolysed proteins. It may be men-

tioned, however, that the same authors are of the opinion that proteins may be heated in 30 % sodium hydroxide for 30 minutes without appreciable loss, whereas Herzfeld showed that with 6 % sodium hydroxide, 10 g. caseinogen, Witte's peptone and haemoglobin respectively produced 0·15, 1·2 and 3·8 mg. of indole. In spite of the evidence to be adduced in this paper, which serves to show that tryptophan is not continuously destroyed during baryta hydrolysis, it is quite possible that at some period there is an appreciable loss, as was suggested by von Fürth. The question must wait final decision until some accurate and reliable method has been devised for estimating tryptophan. The chief point of interest in the following experiments is the comparative stability of tryptophan in baryta, when in the presence of the other products of hydrolysis, compared with the ease with which it is decomposed when alone.

The effect of baryta hydrolysis on the nitrogen content of the mercury precipitate.

An attempt was first made to detect whether there was any loss of tryptophan during the baryta hydrolysis of a protein, by estimating the nitrogen of the mercuric sulphate precipitate. It was thought that, in addition to tryptophan, the mercuric sulphate reagent of Hopkins and Cole [1901] only precipitated cystine, tyrosine, and histidine. Since cystine is destroyed by alkaline hydrolysis, it seemed that, provided tyrosine and histidine were eliminated, the nitrogen content of the mercury precipitate would be a measure of the tryptophan. In the first place it was found that the precipitation of tyrosine can be almost prevented by raising the concentration of the sulphuric acid to 7 % by volume, before adding the mercury reagent, the last traces of tyrosine being easily washed away with 5–7 % sulphuric acid. No tryptophan appears to be removed during this process, especially if the acid for washing contains 2–3 % mercuric sulphate. Moreover, the increased concentration of the acid does not prevent the complete precipitation of tryptophan, even in very dilute solutions, provided the precipitate is allowed to stand from 3 to 4 days, as may be seen from Table I, in which the tryptophan recovered was calculated from the total nitrogen of the mercury precipitate.

Table I. *Influence of the percentage of tryptophan and of H_2SO_4 on the complete precipitation of the mercuric compound.*

Weight of tryptophan taken in mg.	Concentration of tryptophan in mg. %	Concentration of H_2SO_4 %	Weight of tryptophan recovered in mg.	Percentage of apparent loss or gain in mg.
40	20	6·0	39·9	− 0·25
40	20	7·0	39·9	− 0·25
50	20	7·0	49·1	− 1·80
40 (+histidine HCl 0·064 g.)	20	7·0	37·7	− 5·75
40	20	8·0	30·6	−23·50
50	4	7·0	50·1	+ 0·25
50	2	7·0	45·1	− 8·80
50 (+amino-acids 2·5 g.)	2	7·0	50·5	+ 1·00

That the precipitation of tryptophan is facilitated by the presence of other amino-acids is seen by the last experiment, in which 2·5 g. of leucine, and

glutamic and aspartic acids were added to the tryptophan. When alone in 7 % acid, tryptophan is quantitatively precipitated in a concentration of 0·004 %.

Histidine appears to be fairly stable when boiled with baryta, as is shown by the following experiment:

0·5 g. histidine HCl was boiled in 10 % $Ba(OH)_2$ on a sand-bath for 120 hours. The baryta was removed and the solution precipitated with $HgCl_2$ and Na_2CO_3. The total nitrogen of the precipitate was equivalent to 0·345 g. histidine HCl, a loss of 31 %.

Some histidine is precipitated by the mercury reagent, especially in low concentrations of sulphuric acid, and in the presence of the other products of hydrolysis. Even in 7 % sulphuric acid, under the latter conditions, some histidine is precipitated, but this can be avoided by diluting the fluid until it contains only 0·02 % of the base (see Table IV). After having precipitated twice with these concentrations the final product fails to give a sulphur reaction, showing the absence of cystine.

After hydrolysis and the removal of the baryta[1] the caseinogen solutions were twice precipitated with mercuric sulphate, observing the concentrations specified, *i.e.* 7 % H_2SO_4 by volume, and a concentration of histidine not greater than 0·02 %. The total nitrogen of the mercury precipitate from a number of hydrolyses was then estimated, and the results are shown in Table II.

Table II. *Influence of baryta hydrolysis of caseinogen on the nitrogen content of the precipitate obtained with $HgSO_4$ in 7 % H_2SO_4.*

No. of experiment		Weight of substance taken in g.	Period of hydrolysis in hours	Total nitrogen of $HgSO_4$ ppt. in g.	Apparent yield of tryptophan in g.
1	Tryptophan	0·3	60	0·0099	0·072
2	Tryptophan	0·3	120	Traces	—
3	Hammarsten's casein	20·0	40	0·081	0·590
4	"Pure casein*"	20·0	40	0·089	0·649
5	"Pure casein"	20·0	70	0·090	0·656
6	"Pure casein"	20·0	80	0·096	0·700
7	"Pure casein"	20·0	80	0·104	0·758
8	"Pure casein"	20·0	100	0·100	0·729
9	"Laitproto No. 6†"	20·0	40	0·036	0·262
10	"Laitproto No. 6"	20·0	120	0·041	0·299
11	"Pure casein"	20·0	60	0·101	0·736
	glucose	0·2			
12	"Pure casein"	20·0	60	0·088	0·641
	glucose	1·0			
13	"Pure casein" (+ *N* NaOH 13 cc.)	20·0	80	0·051	0·372
14	"Pure casein" (in 10 % NaOH)	20·0	80	0·000	0·000

* "100 % pure casein" purchased from Casein Ltd., Culvert Works, Battersea.

† Commercial sample of caseinogen obtained from the above firm.

[1] A 10 % solution of caseinogen was hydrolysed in 10 % baryta, and some difficulty was encountered in washing the $BaSO_4$ quite free of tryptophan. It was found advantageous to use boiling water containing 10 % of alcohol and a little acetic acid for extracting the precipitate.

These estimations (Kjeldahl) were made in duplicate, and average figures are given throughout. Precautions were taken to add thiosulphate before distillation, and incineration, owing to the resistance of the indole ring [Dakin and Dudley 1914], was continued for an hour after the fluid was quite clear.

From experiments 1 and 2 in Table II it is clear that pure tryptophan is almost completely destroyed when it is boiled in the presence of baryta, the solution turning pink and giving a strong smell of indole as well as a positive vanillin reaction [Nelson 1916]. From experiments 3 to 8, it appears that the nitrogen of the mercuric sulphate precipitate reaches a maximum after about 70 hours, and remains constant after 100 hours' hydrolysis. Samples of commercial caseinogen were found to behave in a very different manner from the pure product, as is shown by experiments 9 and 10, the apparent yield of tryptophan being only 40 % of that of the "pure casein." That this was not due to contamination by traces of lactose is shown by experiments 11 and 12, in which a carbohydrate was added to pure caseinogen without there being any obvious change in the apparent yield of tryptophan. The small yield from commercial caseinogen is due to the large amount of sodium added to these preparations, as may be clearly seen from experiments 13 and 14. For when enough soda is added to a solution of 20 g. of pure caseinogen to make it neutral to phenolphthalein (13 cc. *N* NaOH), the apparent yield of tryptophan is about the same as from the commercial samples. In some preparations, so much alkali is introduced that much of the protein is racemised, and will not digest [Dakin and Dudley 1914]. If pure caseinogen is hydrolysed with 10 % sodium hydroxide, or barium hydroxide containing a sodium salt, no trace of mercury precipitate is obtainable and considerable quantities of indole are produced.

In order to test the effect of baryta hydrolysis on free tryptophan in the presence of the other products of hydrolysis, a number of experiments were carried out with gelatin (which contains no tryptophan) to which were added cystine and histidine to make the total amount of these substances about equal to that in caseinogen. The mercuric sulphate precipitate was prepared under the conditions described above, and the total nitrogen obtained is shown in Table III.

Experiments 1 to 4 show that the nitrogen contained by the mercury precipitate from gelatin is very low, even in the presence of the additional amino-acids, and experiment 5 shows that the same is true of zein. In experiments 6, 7 and 8, after allowing for the 7 mg. of nitrogen from the gelatin, nearly the whole of the tryptophan added is apparently recovered, it being assumed that the presence of the tryptophan does not cause the mercury reagent to precipitate anything extra from the hydrolysed gelatin. From the results recorded in the following communication, however, it is very necessary to put this to the test, as it has been found that the mercury precipitate obtained by the ordinary method for isolating tryptophan contains polypeptides,

Table III. *The nitrogen content of the mercury compound, obtained under the specified conditions, from gelatin and from gelatin + tryptophan, hydrolysed for 80 hours.*

No. of experiment		Weight of substance hydrolysed in g.	Period of hydrolysis in hours	Nitrogen of $HgSO_4$ ppt. in g.	Equivalent weight of tryptophan
1	Gelatin	40·00	80	0·008	—
2	Gelatin	20·00	80	0·006	—
	Cystine	0·05			
3	Gelatin	20·00			
	Cystine	0·20	80	0·008	—
	Histidine	0·50			
4	Gelatin	20·00	80	0·006	—
	Tyrosine	1·00			
5	Zein	15·00	80	0·005	—
6	Gelatin	20·00			
	Cystine	0·20	80	0·041	0·301
	Histidine	0·50			
	Tryptophan	0·30			
7	Gelatin	20·00			
	Cystine	0·02	40	0·037	0·271
	Histidine	0·50			
	Tryptophan	0·30			
8	Gelatin	20·00			
	Cystine	0·02	60	0·044	0·322
	Histidine	0·50			
	Tryptophan	0·30			

the presence of which might raise the nitrogen content of the mercury precipitate, thus compensating for any tryptophan lost. Even allowing for this, it is fair to assume that the presence of other amino-acids in some way protects tryptophan from the destructive action of barium hydroxide. The effect of crystalline amino-acids on the stability of tryptophan was accordingly examined as follows:

The effect of baryta hydrolysis on pure tryptophan in the presence of other free amino-acids.

Tryptophan, with a mixture of tyrosine, glycine, leucine and aspartic acids, was boiled with 10 % baryta for 40 hours, but no smell of indole could be detected. Even a comparatively small amount of a single amino-acid exerts a considerable protective influence.

In flask *A* were placed 5 mg. of tryptophan and some baryta solution. In flask *B* were placed the same amount of tryptophan and 0·125 g. of aspartic acid with the same amount of baryta. After 15 minutes' boiling, neither flask smelt of indole, but after half an hour indole could be detected in flask *A*, and after 45 minutes the contents had turned pink. Flask *B* was boiled for 4½ hours without the appearance of any indole.

A further experiment was carried out to test the protective influence of the amino-acids more rigorously. It was devised so that any compensation for a loss of tryptophan by the precipitation of amino-acids might be detected, thereby serving to show the extent to which tryptophan is destroyed in baryta

hydrolysis. The monoamino-acids added were chosen so as to avoid those which are easily precipitated by mercuric sulphate, and the following were taken:

	g.
Tryptophan (from caseinogen) ...	0·2
Glycine (from gelatin)	1·5
Leucine (from caseinogen) ...	1·3
Alanine (from silk)	4·0
Aspartic acid (from asparagine)	2·0
Glutamic acid (from gluten flour)	1·0
	10·0

This mixture of amino-acids dissolved in 200 cc. of water contained 2 % of tryptophan. Half the solution was boiled for 40 hours with the addition of 5 g. of barium hydroxide. The other half was made up to a volume of 500 cc. containing 5 % sulphuric acid, to serve as a control, and precipitated directly with 150 cc. of the mercury reagent. Some 30–40 cc. of fluid were distilled from the boiled portion, extracted with xylene and tested with vanillin and *p*-dimethylaminobenzaldehyde, both reactions for indole were faint but positive. The distillate, however, gave no precipitate with mercuric sulphate. After the removal of the baryta, the total volume was made up to 500 cc. containing 5 % sulphuric acid, and precipitated with 150 cc. of the mercury reagent. The precipitates were now washed, decomposed with hydrogen sulphide, the mercury removed, and the volume concentrated to 200 cc. Estimations were then made of the total and of the amino-acid nitrogen, which were as follows:

Mixture of amino-acids boiled for 40 *hours with* 10 % $Ba(OH)_2$.

	Nitrogen in g.	Equivalent weight of tryptophan in g.
Nitrogen of tryptophan added ...	0·0137	0·100
Total nitrogen	0·0131	0·095
Amino nitrogen	0·0075	0·110
Non-amino nitrogen (by difference)	0·0056	0·081

Since in the boiled portion the tryptophan calculated from the amino nitrogen is more than the amount taken (0·1 g.), and that calculated from the total nitrogen rather less, it is clear that some other amino-acids have been precipitated with the tryptophan. The actual amount of tryptophan may be calculated from the non-amino nitrogen, since the contaminating amino-acids must be monoamino-acids, and amounts to 0·081 g. a loss of 19 mg. of tryptophan, which may easily be accounted for by the indole-like substance. The amount of monoamino-acids precipitated by the mercury is very small, being equivalent to not more than 2 mg. of nitrogen. In the control experiment 52·3 % of the total nitrogen was found to be amino nitrogen, and in a second control experiment carried out later, 50·2 % of the nitrogen was in the amino form,

showing that very little of the amino-acids was precipitated except tryptophan.

A somewhat similar experiment, but without a control, was carried out with tryptophan, to which were added the products of a 90-hour hydrolysis of gelatin in baryta. The fraction of the hydrolysed gelatin precipitated by $HgSO_4$ was carefully removed, and in addition the remaining histidine was precipitated with $HgCl_2$ and $Ba(OH)_2$. All the reagents were removed, the fluid evaporated to a gum, and 0·2 g. of tryptophan added to 10 g. of these products of hydrolysis. The solution was then boiled for 40 hours in 100 cc. of 10 % $Ba(OH)_2$. The boiled mixture was treated exactly as before, except that the tryptophan was precipitated in a litre of 7 % sulphuric, with 300 cc. of the mercury reagent. The figures obtained for the nitrogen were as follows:

	Nitrogen in g.	Equivalent weight of tryptophan in g.
Nitrogen of tryptophan added ...	0·027	0·200
Total nitrogen	0·031	0·227
Amino nitrogen	0·014	0·207
Non-amino nitrogen (by difference)	0·017	0·245

In this case the tryptophan calculated from the total nitrogen is rather more, and that calculated from the non-amino nitrogen considerably more than the amount added (0·20 g.). This is no doubt due to the large amount of proline and hydroxyproline present in gelatin (over 24 % [Dakin, 1920]), some of which appears in the mercury precipitate, thereby raising the amount of non-amino nitrogen, and consequently the apparent amount of tryptophan. In this case the amino nitrogen is probably a more accurate measure of the tryptophan than the non-amino nitrogen. Boiling produced a small amount of a volatile substance which gave the indole reactions and could moreover be precipitated by mercuric sulphate.

It is an interesting fact that this action of the products of protein hydrolysis in preventing the destruction of tryptophan by barium hydroxide, is the reverse of what is experienced in acid hydrolysis. Miss Homer [1915] found that only 50 % of pure tryptophan is destroyed when it is boiled with 25 % sulphuric acid for 42 hours, whereas when tryptophan is split from the protein molecule, it is destroyed so rapidly that it is only with difficulty that a portion can be recovered.

A 4–5 % solution of caseinogen was hydrolysed with H_2SO_4. One portion was made to contain 5 % sulphuric acid, and another 7 %. After the addition of $HgSO_4$ to both, it was found that though a small precipitate giving the glyoxylic reaction was formed in the 5 % acid, none whatever separated in the 7 % acid, even after standing several days.

Thus the products of hydrolysis seem to protect tryptophan from the action of alkalies, but they assist the destructive action of acids. Further, tryptophan is rapidly destroyed by a univalent ion such as $Na^{\cdot}$, even when it is in the presence of the other products of hydrolysis. The fact that the bivalent $Ba^{\cdot\cdot}$ ion does not rapidly destroy tryptophan when in the presence of amino-acids, etc., is not easily explained; possibly $Ba^{\cdot\cdot}$ can form stable double salts with tryptophan and some other amino-acid, which are not easily decomposed.

The effect on the solubility of amino-acid compounds produced by the presence of other amino-acids in solution.

It has been mentioned that pure histidine monohydrochloride is not easily precipitated in acid solution, yet in the presence of the other products of hydrolysis it is precipitated with considerable ease. This is more clearly demonstrated by Table IV.

Some gelatin was hydrolysed with baryta. The solution was made 5 % acid with H_2SO_4, $HgSO_4$ was added, and the small precipitate filtered off. The excess mercury was removed with hydrogen sulphide, the SH_2 boiled off, and the H_2SO_4 removed quantitatively with baryta. The solution was finally evaporated to dryness, and dissolved in 7 % H_2SO_4 to make a 0·8 % solution, to which histidine was added in varying amounts.

Table IV. *Influence of the products of protein hydrolysis on the solubility of the histidine mercury compound.*

Percentage of histidine in 7 % H_2SO_4	Precipitate after 2 days	Percentage of histidine in 7 % H_2SO_4 + 0·8 % hydrolysed gelatin	Precipitate after 2 days
0·200	–	0·200	+ +
0·100	–	0·100	+
0·050	–	0·050	±
0·025	–	0·025	–

This experiment forcibly illustrated a point familiar in the chemistry of the purines, and repeatedly observed during this work, namely that the ordinary laws of aqueous solubility for amino-acids and their salts, do not apply to amino-acids in solution with each other. The behaviour of the copper salt of tryptophan is a well-known example of this, for though quite insoluble in water, it is easily dissolved by even dilute solutions of other amino-acids. The failure to observe this fact frequently leads to error. Thus Miss Homer [1915] obtained a value for the tryptophan content of caseinogen lower than the amount actually crystallised by the discoverers. This was chiefly due to the use of phosphotungstic acid to remove polypeptides from the solution of tryptophan. Such precipitates always give the glyoxylic reaction.

Van Slyke [1911] observes that three-fourths of the tryptophan nitrogen is precipitated by phosphotungstic acid with the "base fraction." As a matter of fact pure tryptophan is not as a rule precipitated by most samples of phosphotungstic acid, but in the presence of polypeptides, etc., a certain amount is precipitated. Von Fürth and Lieben [1920] claim to separate free tryptophan from that bound as peptides with phosphotungstic acid, by keeping the concentration of tryptophan low and that of the acid high. No proof, however, is given to show that phosphotungstic acid will differentiate between tryptophan and di- or tri-peptides. For instance, the following experiment was tried in which 0·025 g. tryptophan and 0·018 g. of freshly dissolved alanyl-glycyl-glycine were taken in 25 cc. of 5 % HCl, but no matter how much phosphotungstic acid was added, the solution remained clear indefinitely.

Conclusions.

1. When caseinogen is hydrolysed in acid solution, tryptophan is rapidly destroyed. When pure tryptophan is boiled in acid solution, it is much more stable.

2. Tryptophan when alone is rapidly destroyed by all alkalies, but NaOH and Na_2CO_3 are more effective than $Ba(OH)_2$.

3. When caseinogen is hydrolysed by NaOH, tryptophan is rapidly destroyed, but when $Ba(OH)_2$ is used, there is evidence to show that tryptophan is much more stable.

4. The destruction of tryptophan by the univalent, but not by the bivalent ion, seems to depend on the presence of the other products of hydrolysis. The destruction of tryptophan may be largely prevented by the addition of crystalline amino-acids.

5. The precipitation of free tyrosine by the mercury reagent may be prevented to a very great extent by raising the concentration of the H_2SO_4 from 5 to 7 % by volume.

6. Histidine is fairly stable when boiled with baryta. In acid solutions it is not easily precipitated by $HgSO_4$ except in high concentrations, but in the presence of other amino-acids it is precipitated in 7 % H_2SO_4, unless its concentration is reduced to 0·02 %.

7. The total nitrogen of the $HgSO_4$ precipitate, obtained under the specified conditions from hydrolysed caseinogen, reaches a maximum in 60–70 hours, and does not decrease after 100 hours.

8. Gelatin and zein, after baryta hydrolysis, and the specified treatment, yield only traces of nitrogen in the $HgSO_4$ precipitate. After the addition of pure tryptophan to gelatin the total nitrogen of the $HgSO_4$ precipitate appears equivalent to the tryptophan added.

References.

Dakin (1920). *J. Biol. Chem.* **44**, 499.
Dakin and Dudley (1914). *J. Biol. Chem.* **17**, 275.
von Fürth and Lieben (1920). *Biochem. Zeitsch.* **109**, 153.
Herzfeld (1913). *Biochem. Zeitsch.* **56**, 82.
Homer (1915). *J. Biol. Chem.* **22**, 369.
Hopkins and Cole (1901). *J. Physiol.* **27**, 418.
Nelson (1916). *J. Biol. Chem.* **24**, 528.
Van Slyke (1911). *J. Biol. Chem.* **10**, 40.

XLIV. ON THE NATURE OF THE SUBSTANCES PRECIPITATED BY MERCURIC SULPHATE FROM HYDROLYSED CASEINOGEN, WITH REFERENCE TO THE ESTIMATION AND ISOLATION OF TRYPTOPHAN.

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In the preceding communication [Onslow, 1921] a procedure was described, by which the mercuric sulphate precipitate from hydrolysed caseinogen could be prepared, containing no free cystine, tyrosine or histidine. It was hoped that this precipitate would consist entirely of the tryptophan compound, which might be used as the basis of a method for estimating tryptophan. The nitrogen content, however (calculated for ash- and water-free caseinogen), corresponded to 4 % of tryptophan[1], which suggested that other amino-acids were precipitated, possibly in the form of polypeptides.

Redigestion of the mercury precipitate owing to the difficulty of effecting complete hydrolysis of the peptides.

To investigate the nature of the precipitate an attempt was first made to use Dakin's method [1918] for extracting tryptophan with butyl alcohol, using caseinogen which had been hydrolysed by baryta as previously described. Although Dakin obtained 1·7 % of tryptophan, very little was found on this occasion. It was impossible by extraction to free the fluid from the glyoxylic reaction. Moreover, when the butyl alcohol was evaporated under reduced pressure, a pigmented substance remained as a gum, while little or no tryptophan crystallised[2]. Further experiments were made, in which caseinogen was hydrolysed by a pancreatic extract instead of by baryta, and the resulting mercury precipitate after decomposition was extracted with butyl alcohol. Only a little

[1] The molecular weight of caseinogen was calculated by Van Slyke and Bosworth [1913] as 8888. This would give 2·3 % of tryptophan, supposing that each molecule of the protein contained one molecule of tryptophan. The most recent colorimetrical methods for estimating tryptophan, though very inadequate, give values for caseinogen of about 1·7–2 %.

[2] An attempt was also made to crystallise the racemic sodium salt of β-naphthalenesulphotryptophan from the products extracted by butyl alcohol, but even allowing for the usual yield of 84 %, the tryptophan obtained only amounted to 1 % of the caseinogen taken.

more tryptophan was obtained than before, and it was concluded that most of it must be present as polypeptides, which would dissolve in the butyl alcohol and leave a gum after evaporation. This appeared all the more probable, because Dakin obtained over 4 % of peptide anhydrides, after 14 hours acid hydrolysis, and von Fürth and Lieben [1920] have since shown that even after prolonged pancreatic and ereptic digestion, about two-thirds of the tryptophan remains in the peptide form.

In order to obtain the maximum amount of hydrolysis, the decomposed mercury precipitate was subjected to a second tryptic digestion, in the absence of the other products of hydrolysis, and again extracted with butyl alcohol. The tryptophan *now* separated in an amount hardly inferior to that obtained by Dakin. It was, however, necessary to extract the fluid for a long time before the glyoxylic reaction became negative, but the amount of nitrogen still remaining in the extracted fluid indicated that a quantity of dicarboxylic, or diamino-acids had been thrown down in the mercury precipitate.

Since all the tryptophan was not obtained in a free state by this treatment, some caseinogen was first digested with pepsin, then with trypsin at a reaction of $P_H = 8{\cdot}1$[1], and finally, with erepsin at the same reaction.

The mercury compound obtained was washed and decomposed as usual, and the solution again digested with trypsin and erepsin. In one or two experiments, in which digestion had been complete, the fluid obtained from the decomposed mercury compound would not give the reaction for polypeptides described by Kober [1911]. This is carried out by making the copper salts of the mixture. The copper of all the amino-acids is precipitated by boiling in weak alkaline solution, whereas the copper of the peptides remains unchanged, appearing as a blue filtrate. As a rule, however, it was impossible to obtain complete hydrolysis, and this agrees with the observations of Frankel [1917], who used Van Slyke's method to estimate the amino nitrogen. He found that after digesting proteins 100 hours with pepsin, they gave 20 % of amino-acid nitrogen, with pepsin and trypsin 70 %, with trypsin alone 50 %, and with pepsin, trypsin and erepsin 80–90 %. The difference between these results and those of von Fürth depends apparently on the fact that the latter removed the polypeptides with phosphotungstic acid, so that their amino nitrogen could not react during the formaldehyde titrations.

The amino-acids precipitated by the mercury reagent.

It was found necessary to identify as many as possible of the amino-acids in the mercury precipitate, and for this purpose the usual methods for separating them were adopted. The exact procedure can be followed most easily by reference to the scheme given below, in which the successive operations are shown in tabular form. The amino-acids could not be subjected to Fischer's

[1] According to various authors the optimum reaction for digestion of caseinogen by trypsin is about $P_H = 7{\cdot}2$, but according to a recent investigation by S. W. Cole [1920] maximum digestion occurs at $P_H = 8{\cdot}1$.

ester method owing to the small quantities available, consequently it was impossible to isolate some of the units.

Experiment I. A sufficient amount of the mercury compound was prepared by digesting 1 kilo. of caseinogen with trypsin and precipitating with mercuric sulphate. This precipitate, after being washed free from tyrosine, was decomposed and again well digested with trypsin, in order to carry the hydrolysis of the peptides as far as possible.

Table showing the procedure followed in separating the amino-acids.

(1) 10 % solution of caseinogen digested with trypsin, precipitated in 5% H_2SO_4 with $HgSO_4$, and filtered.

(2) Precipitate* decomposed with SH_2, redigested with trypsin, and again precipitated with $HgSO_4$ in 7 % H_2SO_4, and filtered.

<table>
<tr><td>Precipitate A.
(Contains tryptophan, etc.)

Discarded</td><td colspan="4">Filtrate B.
(Contains amino-acids liberated by second digestion)

Excess mercury removed with SH_2. Fluid precipitated with phosphotungstic acid in 5 % H_2SO_4, and filtered</td></tr>
<tr><td></td><td colspan="2">Precipitate C.
(Contains diamino-acids)

Precipitate decomposed with hot baryta, excess barium removed and histidine precipitated with $HgCl_2$ and Na_2CO_3; filtered</td><td colspan="2">Filtrate D.
(Contains monoamino-acids)

Excess phosphotungstic acid and H_2SO_4 removed. Dicarboxylic acids precipitated by $Ca(OH)_2$ and alcohol (Foreman [1914]); filtered</td></tr>
<tr><td></td><td>Precipitate E.

Precipitate of histidine decomposed by SH_2 and picrolonic acid added to isolate the HISTIDINE, which was identified by melting-point and N-content</td><td>Filtrate F.

Excess Hg removed. Phosphotungstic acid added in 5 % H_2SO_4. No precipitate of LYSINE or ARGININE</td><td>Precipitate G.

Precipitate of Ca salts dissolved in H_2O. Ca removed with oxalic acid and $Cu(OH)_2$ added to isolate ASPARTIC and GLUTAMIC acids</td><td>Filtrate H.
(Contains monoamino-acids)

Alcohol evaporated and residue allowed to crystallise. Deposited LEUCINE and traces of TYROSINE. Other amino-acids remained which would not crystallise</td></tr>
</table>

* This precipitate after decomposition gave a faint reaction for sulphur showing the presence of cystine.

From precipitate *G* crystals of what appeared to be a mixture of the copper salts of aspartic and glutamic acids were obtained, but no attempt was made to isolate hydroxyglutamic acid.

The other monoamino-acids contained in filtrate (*H*) were allowed to crystallise slowly, typical striated nodules of impure leucine being deposited. These were dissolved and the fluid recrystallised from traces of tyrosine. The resulting product sublimed on heating, but the amino nitrogen value was lower than that of pure leucine. The filtrate still contained other monoamino-acids which would not crystallise, and as the material was very scarce, an

unsuccessful attempt was made, at the suggestion of Mr Raistrick, to separate the remaining acids as the picrates of the methyl ester hydrochlorides.

Experiment II. In order to make quite certain that arginine and lysine are not precipitated in the mercury compound, the diamino-acids were treated according to the method of Kossel and Kutscher. For this purpose 500 g. of caseinogen were digested and treated as in Experiment I, until the filtrate (*B*) had been obtained. The arginine and histidine were then precipitated as silver salts in a solution made alkaline with baryta and filtered off. The filtrate was tested for lysine with phosphotungstic acid which failed to give any precipitate. The arginine-histidine fraction was next decomposed, the histidine separated as a silver salt in neutral solution, and the filtrate tested for arginine by saturating it with baryta. No precipitate could be obtained, thus confirming the absence of arginine and lysine.

Experiment III. In order to find out whether proline was present among the monoamino-acids, a further 200 g. of caseinogen were digested and precipitated with the mercury reagent. Special pains were taken to ensure that this precipitate was washed free from all tyrosine. This was done by grinding the precipitate with 5 % sulphuric acid until the filtrate gave a negative Millon's reaction. The washing was then continued until 200 cc. of the filtrate, after being freed from mercury and sulphuric acid, failed to give the reaction for tyrosine with diazobenzenesulphonic acid [Totani 1915]. It was observed that after the tyrosine had been completely removed, a strong histidine reaction could still be obtained.

The washed mercury precipitate was now decomposed and boiled for 14 hours in 25 % sulphuric acid, to hydrolyse the peptides. The resulting black fluid was diluted until it contained 6 % of acid. Enough baryta was added to reduce the concentration to 5 % and the barium sulphate, together with most of the humin, filtered off. The filtrate was precipitated with mercuric sulphate, to separate the tryptophan, and the filtrate, after removing the reagents, was treated according to Dakin's method [1918] for preparing proline, by thoroughly extracting with butyl alcohol. The alcohol was concentrated to remove the monoamino-acids which separate, and the filtrate after taking to dryness under reduced pressure was dissolved in absolute alcohol and set aside to crystallise. The traces of peptide anhydrides deposited were filtered off, and the residue after taking to dryness was boiled in water with a little Merck's charcoal. As no more peptide anhydrides separated, the fluid was made up to a known volume, and the total and amino nitrogen estimated in duplicate, with the following result: amino nitrogen by Van Slyke, 3·1 mg.; total nitrogen by Kjeldahl, 13·1 mg.; non-amino nitrogen by difference, 10 mg.; equivalent weight of proline, 82 mg. It thus appears that there is a very small but definite amount of proline precipitated in the mercury compound which amounts to about 82 mg. from 200 g. of caseinogen.

It was impossible to overlook the fact that arginine or lysine might have been absent from filtrate (*B*) in experiments I and II, because they were

precipitated with the tryptophan in precipitate (*A*). In order to decide this, advantage was taken of the fact that nearly all the polypeptides must have been hydrolysed by boiling with strong acid, thus insuring the most complete separation possible between the tryptophan and the other amino-acids. In addition proline and the other monoamino-acids had been removed as described above, so that the aqueous layer after extraction with the butyl alcohol must contain all the dicarboxylic acids and the bases. This fraction was accordingly treated with mercuric chloride and sodium carbonate to precipitate the histidine. After removing the reagents, phosphotungstic acid was added to precipitate any lysine or arginine. No precipitate resulted, except after long standing, and this gave a strong histidine reaction, showing that this amino-acid had not been completely removed by treatment with mercuric chloride.

As there was some doubt whether the trace of tyrosine observed in filtrate (*H*) of experiment I, was due to the precipitation of a tyrosine polypeptide, or to the incomplete washing of the mercury precipitate from free tyrosine, great pains were taken to wash the precipitate thoroughly, as already described. Advantage was now taken of this precaution, to test for tyrosine in the fraction of the monoamino-acids extracted by butyl alcohol[1]. Tyrosine was present only in the peptide anhydrides separated from the ethyl alcohol, showing that a minute quantity of tyrosine peptide is precipitated with the mercury compound. This cannot be washed away with acid and appears to be hydrolysed with great difficulty.

As the result of the foregoing experiments, it may be concluded that the mercuric sulphate compound contains the following amino-acids in addition to tryptophan: cystine, tyrosine (traces), leucine and other monoamino-acids, glutamic and aspartic acids, histidine and a little proline, but neither lysine nor arginine. It is probable that most of these amino-acids are precipitated or adsorbed in the peptide form, possibly as tryptophan polypeptides. The amount of amino-acids precipitated by the mercury depends on the degree of hydrolysis. It was frequently noticed, especially when digestion was incomplete, that the mercury compound was precipitated in two layers, the first yellow, the other white. The latter possibly consisted mainly of polypeptides.

Is tyrosine split from the protein molecule early in digestion?

The fact that tyrosine was recognised in the peptide form after hydrolysis of a small fraction of the total protein in 25 % sulphuric, made it doubtful whether this amino-acid is split from the protein molecule as early as is sometimes claimed. Plimmer and Eaves [1913] have based a method of estimating

[1] It is impossible to test the decomposed mercury precipitate directly for tyrosine because the tryptophan present interferes with Millon's reagent, and the histidine masks the colour of the diazo reaction. The phenol reagent of Folin and Denis [1912] is said to give a blue colour with tyrosine alone of the protein decomposition products. Although this reagent was tried, it was found useless, since it gave a blue colour with the purest samples of tryptophan procurable. Abderhalden and Fuchs [1913] and Gortner and Holm [1920] have since shown that tryptophan and other amino-acids also give this blue colour.

tyrosine on the assumption that in pancreatic digestion this amino-acid is yielded quantitatively within six hours. Totani [1916] isolated 0·174 g. of tyrosine by acid hydrolysis after the removal of all the tyrosine formed during digestion, that is to say, nearly 0·3 % of the caseinogen taken. The tenacity with which tyrosine remains bound in the peptide linkage was therefore tested in the following way:

10 g. of caseinogen were digested for 6 hours, as directed by Plimmer and Eaves. The polypeptides, etc., were precipitated with phosphotungstic acid and after decomposition with baryta and the removal of the excess Ba, the filtrate was submitted to a thorough redigestion. After concentrating, 0·137 g. of a mixture of tyrosine and leucine was obtained, which on recrystallisation yielded 0·02 g. of pure tyrosine, or 0·2 % of the caseinogen taken. The filtrate still gave a strong Millon's reaction.

The preparation of tryptophan by the use of butyl alcohol.

It will be recollected that extraction of the decomposed mercury precipitate with butyl alcohol, as described by Dakin, only gave very small yields of tryptophan, not more than 0·2–0·5 %, which is the average yield of the Hopkins and Cole method. If, however, the decomposed mercury precipitate was subjected to a second digestion in the absence of the other products of hydrolysis, much larger yields of an almost pure product were secured. Owing to the great difficulty usually experienced in securing a good yield of tryptophan, a method by which 1·3–1·7 % may consistently be obtained by any laboratory assistant of ordinary skill, appears worthy of description.

Digestion of the caseinogen. To prevent the rise of the hydrogen ion concentration due to the liberation of the amino-acids, it is advisable to digest the caseinogen in a buffer solution. This solution is best prepared as follows: to 10 litres of tap water in a large vessel add 136 g. of crystalline sodium acetate, and gradually stir in 1 kilo. of caseinogen[1]. To this suspension add strong sodium hydroxide, until a drop of the mixture reacts alkaline to cresol red (*o*-cresol-sulphon-phthalein) and acid to phenolphthalein. The P_H will then be somewhere close to 8·1. Next add 15–20 g. trypsin[2], 20 g. sodium fluoride and 50 cc. of toluene. Fill into Winchester quart bottles and place in an incubator at a temperature of not less than 38°. If crystals of tyrosine do not form on the sides of the bottles after three or four days, add more enzyme and return to the incubator.

Separation of the mercuric sulphate compound. When digestion is complete filter the fluid with the aid of kieselguhr on several large Buchner funnels acidify the filtrate with 13 cc. of ice-cold 50 % sulphuric acid to every 87 cc. of the fluid, and add 120–150 cc. of the mercuric sulphate reagent to

[1] "Laitproto No. 6," sold by Casein Ltd., Culvert Works, Battersea, is a fairly good commercial product, but it is important to remember that, owing to treatment with alkali, many commercial samples are racemised to such an extent that they will only partially digest.

[2] An active ferment is sold by the British Drug Houses as "trypsin," prepared by precipitation with ammonium sulphate. A less active preparation precipitated with alcohol is sold as "pancreatin." Excellent results may also be obtained with an acid extract of the pancreas [Cole 1920], or even by adding the minced gland itself.

every litre. Allow the precipitate to stand overnight, filter off, wash with 7 % H_2SO_4 until the filtrate no longer gives a Millon's reaction, and then with water. Suspend in a litre of water, add a hot saturated solution of baryta (about 20 g. in 50 cc. of water) until distinctly alkaline, and decompose with SH_2. Filter off the mercury sulphide and remove the SH_2 under reduced pressure, bring the fluid to a reaction of about $P_H = 8{\cdot}1$ by the addition of baryta or sulphuric acid, and digest with trypsin for the second time (5 to 7 days). Neutralise to litmus and concentrate under reduced pressure to 150–200 cc.

Extraction with butyl alcohol. It is preferable to carry out the extraction with butyl alcohol[1] in a stout flask rather than in a continuous extraction apparatus, so that the alcohol may be concentrated under reduced pressure. The flask should be fitted with two corks, one of which is supplied with a "pipette" similar to that used for blowing off the ether in Meig's method of fat extraction [see Cole, 1920]. Shake the flask intermittently and keep it at a temperature of about 70° on the water-bath. Blow off the hot, supernatant layer of alcohol into a Claisen flask prepared for distillation under reduced pressure, and repeat the extraction with a second volume of butyl alcohol. In the meanwhile, the distillate from the Claisen flask has separated into two layers, since the alcohol is more soluble in hot than in cold water. For the third extraction the alcoholic layer only should be used, thus obtaining a gradual reduction in the volume of the fluid extracted. Most of the tryptophan will have separated after 10–15 extractions carried out in this way. Reduce the alcohol in the Claisen flask to 40–50 cc. and filter off the tryptophan which at first usually crystallises in slightly discoloured shining plates. A second and third crop may be obtained by concentrating still further, but when the tryptophan begins to separate in a gummy condition, it is advisable to drive off the rest of the alcohol, to dissolve the residue in water, and to reserve it together with the extracted fluid for the next preparation, when it is added to the main portion just before precipitation with the mercury reagent. The amount of tryptophan obtained should be 10–15 g. which may be rendered pure by boiling in 60 % ethyl alcohol with a little Merck's charcoal, and recrystallising. Estimation of the amino nitrogen in Van Slyke's micro apparatus gave: amino N found, 6·93 %; calculated, 6·86 %.

Conclusions.

1. The mercuric sulphate compound precipitated from hydrolysed caseinogen was found to contain a number of amino-acids, probably combined with tryptophan as polypeptides. After removal of the free tyrosine, the following amino-acids were identified: leucine, cystine, tyrosine (traces) and other monoamino-acids; glutamic and aspartic acids, histidine and a little

[1] Owing to the ease with which acetone and aldehydes destroy tryptophan, the commercial butyl alcohol should be shaken with a saturated solution of sodium bisulphite, and fractionated, the procedure being repeated three times.

proline but neither lysine nor arginine. The degree of hydrolysis no doubt regulates the quantity of peptides precipitated, and possibly, to some extent, even the nature of the individual amino-acids.

2. The presence of combined tyrosine after severe acid hydrolysis indicates that this amino-acid does not separate quantitatively as early in digestion as is sometimes assumed.

3. It is difficult to secure digestion of caseinogen sufficiently complete to yield 1·7 % of free tryptophan, except by redigestion of the decomposed mercury precipitate. After this procedure, however, extraction with butyl alcohol results in a yield of tryptophan which compares favourably with that obtained by Dakin. A detailed account of the procedure for preparing tryptophan by this method is given.

REFERENCES.

Abderhalden and Fuchs (1913). *Zeitsch. physiol. Chem.* **83**, 468.
Cole (1920). Practical Physiological Chemistry, 211 (Heffer, Cambridge).
Dakin (1918). *Biochem. J.* **12**, 290.
Folin and Denis (1912). *J. Biol. Chem.* **12**, 245.
Foreman (1914). *Biochem. J.* **8**, 463.
Frankel (1917). *J. Biol. Chem.* **26**, 31.
von Fürth and Lieben (1920). *Biochem. Zeitsch.* **109**, 153.
Gortner and Holm (1920). *J. Amer. Chem. Soc.* **42**, 1678.
Kober (1911). *J. Biol. Chem.* **10**, 9.
Onslow (1921). *Biochem. J.* **15**, 383.
Plimmer and Eaves (1913). *Biochem. J.* **7**, 297.
Totani (1915). *Biochem. J.* **9**, 385.
—— (1916). *Biochem. J.* **10**, 382.
Van Slyke and Bosworth (1913). *J. Biol. Chem.* **14**, 227.

XLV. A METHOD FOR THE COLORIMETRIC ESTIMATION OF CARNOSINE.

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Part of a Thesis approved for the Degree of Master of Science in the University of London.

The idea of estimating carnosine colorimetrically is based on the fact that histidine when treated with diazobenzenesulphanilic acid and sodium carbonate gives rise to an intense red colour and this led to an attempt by v. Fürth and Hryntschak [1914] to evolve a colorimetric estimation of carnosine nitrate.

They took the base prepared according to the method of Gulewitsch and precipitated it as a copper salt. A known amount was then suspended in water and decomposed with hydrogen sulphide. Using the colorimetric method of Weiss and Ssoblew [1914] they then compared the intensity of the red colour obtained on diazotising the carnosine solution with that given by a standard histidine solution when treated in the same way. The results were:

0·195 g. carnosine copper salt gave 0·173 g. carnosine (theoretically 0·145 g.).

0·6378 g. carnosine copper salt gave 0·430 g. carnosine (theoretically 0·473 g.).

From this it may be seen that the method was decidedly inaccurate and could not be used with confidence if the amount of carnosine were unknown.

These observers then worked on extracts of horse flesh, precipitated the carnosine as the copper salt, decomposed it, and then, by colorimetric means, obtained an average of 0·27 % in the extract. Dietrich [1914], however, had by precipitation methods found the percentage to be 0·17. The discrepancy was possibly due to the numerous precipitations involved, each probably being a source of loss of carnosine.

In 1919 Koessler and Hanke [1919] described a colorimetric method for estimating iminazole derivatives based on the diazo-reaction. The chief modification was the substitution of the standard histidine solution by a standard consisting of a mixture of methyl orange and Congo red (CR . MO), which gave a colour matchable with diazotised histidine.

Since their method had been worked out for histidine, histamine and other iminazole derivatives, it was probable that it could be used for the direct estimation of carnosine in muscle extracts.

Experimental method used.

The colorimetric estimation of carnosine to be described was, accordingly, based on Koessler and Hanke's method for the estimation of pure solutions of iminazole compounds. Several modifications were found necessary before the method could be used to determine the amount of carnosine present in a muscle extract, which naturally is more complex in composition than a pure iminazole solution and contains substances which inhibit the diazo-reaction.

The modifications introduced were as follows:

(1) *Modification with regard to mixing reagents.*

The first alteration was in regard to the mixing of the reagents.

Koessler and Hanke [1919] mixed their solutions directly in the colorimeter cup and laid stress on the necessity for this procedure. Using their suggested amounts, however, it was found impossible to set the test cylinder at the proposed depth of 20 mm. since the colorimeter was larger than theirs.

Double quantities of the reagents were thoroughly mixed in a beaker and were then transferred to the colorimeter cup. No difference in the final colour was obtained whether the reagents were mixed in the cylinder or in the beaker. The depth of colour was also unaltered whether the mixture was transferred directly after mixing in the beaker or at any other stage up to the development of a maximum red colour.

The temperature of the laboratory, within ordinary limits, was also without effect on the reaction. Estimations carried out in winter and in summer gave parallel results, as also did those performed with the mixing beaker near a Bunsen flame.

(2) *Modification with regard to linear relation of readings.*

Koessler and Hanke obtained a perfect linear relation between the amount of histidine diazotised and the readings of the standard CR . MO cylinder.

Thus they stated that:

0·00002 g.	histidine	matched at	10	mm.
0·00003 ,,	,,	,,	15	,,
0·00004 ,,	,,	,,	20	,,
0·00005 ,,	,,	,,	25	,,

On attempting to repeat the results of these observers, no such linear relation could be found. The depth of colour formed by diazotising histidine and carnosine was not proportional to the amount of solution diazotised, though for any given amount the readings were constant.

Some actual figures obtained using a standard solution of histidine matched against a CR . MO indicator solution were:

Amount of iminazole diazotised	Actual reading of standard cylinder	Theoretical reading assuming linear relationship
0·00002 g. histidine	10·75 mm.	10·75 mm.
0·00003 ,, ,,	18·70 ,,	16·10 ,,
0·00004 ,, ,,	28·1 ,,	21·5 ,,
0·00005 ,, carnosine	13·7 ,,	13·7 ,,
0·0001 ,, ,,	30·0 ,,	27·4 ,,

From these results found experimentally it was decided that linear relationship between depth of colour and amount of carnosine diazotised could not be accepted. It was therefore decided to base all results on a constant reading of the standard cylinder.

In all cases the amount of solution diazotised was altered till, when set at 20 mm., it gave a colour matching the indicator cylinder when set at 30 mm. This reading was chosen since a match with the indicator at 30 mm. corresponded to 0·0001 g. of carnosine and calculations were simplified.

(3) *Number of extractions needed.*

Three portions of a sample of English veal were taken and treated as follows:

(*a*) Extracted once with 50 cc. of water; yield 1·15 % carnosine.

(*b*) Extracted twice with two separate 50 cc. water; yield 1·12 % carnosine.

(*c*) Extracted three times with three separate 50 cc. water; yield 1·15 % carnosine.

With three portions of rabbit muscle treated similarly the results were 0·15, 0·15, 0·14 % respectively.

Accordingly, for further estimations it was considered that one extraction was sufficient to give a full yield of carnosine.

This fact indicates that carnosine is in very loose combination with the muscles and the possibility that the substance estimated is histidine and not carnosine is practically ruled out since acid hydrolysis is needed to split histidine from a protein.

(4) *Modification with regard to the presence of proteins.*

Since proteins had been found by Koessler and Hanke to lower the readings of a standard histidine solution by 30 % it was obviously necessary to obtain a precipitant for soluble proteins when estimating carnosine in a watery extract of muscle.

Alcohol and ammonium sulphate cause the formation of an unmatchable yellow colour if added to histidine or carnosine before diazotising and therefore could not be used to free the extracts from protein.

Potassium dihydrogen phosphate up to a concentration of 5 % was stated by Koessler and Hanke to cause no alteration in the colour of a diazotised histidine solution. This led to a trial of metaphosphoric acid as a protein precipitant according to the method of Folin and Denis [1916].

A solution of carnosine was made of such a strength that when 1 cc. was diazotised and set at 20 mm. it gave a match with the CR . MO at 25 mm.

30 cc. of this solution were taken, 10 cc. of 20 % metaphosphoric acid added and the mixture was allowed to stand for three hours.

No precipitate was formed and therefore the metaphosphoric acid removes no carnosine from the solution.

The acid carnosine solution, which it was unnecessary to filter, was then made neutral to litmus with 10 % caustic soda and made up to 50 cc. with distilled water.

1·62 cc. of this solution gave a match with the indicator at 25 mm. against the theoretical 1·66 cc.

This experiment showed that metaphosphoric acid has no precipitating effect on carnosine and that it does not affect the red colour of diazotised carnosine.

It then remained to prove that metaphosphoric acid would successfully remove proteins from a solution without carrying down carnosine as an adsorption compound, or in any other physico-chemical manner.

A 5 % solution of egg albumin was made up and found to give a strong yellow colour with the diazo-reagent. No trace of red was present. To 30 cc. of this protein were added 50 cc. of the carnosine solution previously used (*i.e.* 1 cc. when diazotised gave a match with the CR . MO at 25 mm.) and 10 cc. of 25 % metaphosphoric acid. After standing overnight the carnosine protein mixture was filtered, neutralised with 10 % caustic soda and made up to 100 cc.

2 cc. of this filtrate gave a match with the CR . MO at 24·9–25·1 mm.

Since the mixture was 50 % carnosine and 50 % albumin, acid and water, 2 cc. equalled the original 1 cc. which on treatment gave a match at 25 mm.

Similar results were obtained using gelatin and blood albumin as the protein added to the carnosine solution.

These experiments showed that metaphosphoric acid effectually removed the proteins without in any way interfering with the diazotisation of carnosine. They also showed that metaphosphoric acid does not split off histidine from the proteins when used as a precipitant, since the result on diazotising a standard carnosine protein solution was unaltered by precipitation with metaphosphoric acid.

A further test was made using metaphosphoric acid to precipitate the proteins in "Marmite," a commercial preparation of yeast.

The resultant filtrate when diazotised gave a deep yellow colour without a trace of the typical carnosine red.

A standard carnosine solution was taken of which 1 cc. gave a match with the indicator at 29·95 mm. 13·9 g. of "Marmite" were dissolved in 30 cc. of water. 50 cc. of the carnosine solution were added, together with 10 cc. of 20 % metaphosphoric acid. This mixture was allowed to stand overnight. It was then filtered, neutralised to litmus and made up to 100 cc.

2 cc. of this filtrate (*i.e.* the same amount of carnosine as previously gave a reading of 29·95 mm.) gave a match at 29·95–29·97 mm. The additional yellow tinge caused by the diazo-reagent on the "Marmite" gave a more perfect match against the CR . MO standard than when pure carnosine was diazotised.

Similar results were obtained when cod extract was used in place of "Marmite." 13·96 g. of cod were extracted with 50 cc. of water and 10 cc. of 25 % metaphosphoric acid were used to precipitate the protein.

After filtration and neutralisation 40 cc. of the mixture were diluted to 100 cc. On diazotisation no pink colour was formed, but again a deep yellow resulted.

A further 14 g. of cod muscle was treated in exactly the same way, except that besides the water and acid 60 cc. of carnosine solution were added. This carnosine was of such a strength that 1 cc. when diazotised gave a match with the CR . MO at 27·8–28·2 mm.

The fish-carnosine mixture gave a match with the CR . MO at 28·0–28·3 mm. when 2 cc. of the filtrate were treated with diazobenzenesulphanilic acid and sodium carbonate.

Here again there was no difference in the colour obtained when proteins in the solution were first precipitated by means of metaphosphoric acid, though when free in the diazotised liquid they decreased or even destroyed the colour formation.

It was also obvious that no histidine was liberated from commercial protein or fresh cod muscle by the experimental methods used, otherwise on diazotisation a deeper red colour than could be accounted for by the added carnosine would have been obtained.

Metaphosphoric acid neither destroys nor interferes with the reaction and the proteins are effectually removed by it, since the final solution gives negative results with Millon's, the xanthoproteic and glyoxylic tests. In carnosine-free solutions the biuret reaction is also negative.

Therefore it was decided that metaphosphoric acid up to a concentration of 10 cc. of 20 % solution per 50 cc. of fluid has no effect on the colour formed by adding diazobenzenesulphanilic acid and sodium carbonate to a carnosine solution.

Final method adopted.

The final method adopted for the colorimetric estimation of carnosine in muscle extracts is as follows:

A weighed amount of finely minced tissue is extracted for 15–60 minutes at 60–90° in a known volume of water. This is then filtered through a dry paper into a dry beaker and the pulp well squeezed out. 2–10 cc. of 20 % metaphosphoric acid (according to the amount of tissue taken) are added and the mixture is left to stand 2–24 hours.

The solution is then filtered from the precipitated protein. An aliquot part of the filtrate is placed in a graduated flask, neutralised to litmus with 10 % caustic soda and made up to the mark with distilled water. This neutralised solution is treated as a carnosine solution. 10 cc. of 1·1 % sodium carbonate are placed in a clean dry beaker together with 2–X cc. of distilled water, 4 cc. of the diazo-reagent are run in from a pipette, and the whole

mixed by shaking the beaker. Exactly one minute later X cc. of the extract are added.

After mixing the diazotised solution is placed in one cup of a Duboscq colorimeter which is then set at 20 mm. On the other side is Koessler and Hanke's [1919] CR. MO standard. This standard cylinder is moved till it matches the unknown when the colour of the latter has gained its full intensity (about 5 minutes from mixing). Readings are taken and the value of X altered till, with the unknown set at 20 mm., the CR. MO cylinder matches at 30 mm.

As this is so when $X = 1$ cc. of a 0·01 % carnosine solution, the amount of extract required to give this colour is taken to contain 0·0001 g. carnosine.

An actual experiment was as follows:

13·56 g. rabbit muscle were extracted with 50 cc. of distilled water at 60° for 25 minutes.

The solution was then filtered and the pulp squeezed out. To the filtrate were added 10 cc. of 20 % metaphosphoric acid. The mixture was allowed to stand 24 hours and was then filtered.

30 cc. of this filtrate were taken, neutralised with 10 % soda, and made up to 100 cc. in a graduated flask.

Different amounts were then diazotised and compared with the CR. MO indicator.

$X = 1{\cdot}0$ cc. gave a match with the indicator cylinder at 30 mm., whereas 0·54 was insufficient and 1·5 cc. too strong.

Therefore 1 cc. contained 0·0001 g. of carnosine.

100 „ „ 0·01 „ „

And since half the original filtrate was taken the 100 cc. contained half of the carnosine of the extracted muscle.

Therefore percentage $= \frac{2 \times 0{\cdot}01 \times 100}{13{\cdot}6} = 0{\cdot}15$ % carnosine.

In another experiment 8·5 g. lean imported beef treated in a similar manner gave 0·36 % carnosine.

Parallel estimations should agree to 0·05 mm. Thus in the above experiment the readings obtained for separate amounts of 0·94 cc. each were:

29·96, 30·0, 29·98, 29·99, 29·96, 30·1, 30·0.

This method has been used for over one hundred estimations of carnosine in various muscles and is the basis of an investigation now in progress of the distribution of carnosine in the animal kingdom.

SUMMARY.

A modification of Koessler and Hanke's colorimetric method for estimating iminazole derivatives has been devised which applies to the estimation of carnosine.

Thanks are due to Professor E. Mellanby for suggesting the work and for help in its progress, also to Professor V. H. Mottram for his help in this research.

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REFERENCES.

Dietrich (1914). *Zeitsch. physiol. Chem.* **92**, 212.
Folin and Denis (1916). *J. Biol. Chem.* **26**, 491.
v. Fürth and Hryntschak (1914). *Biochem. Zeitsch.* **64**, 172.
Koessler and Hanke (1919). *J. Biol. Chem.* **39**, 497.
Weiss and Ssoblew (1914). *Biochem. Zeitsch.* **58**, 119.

XLVI. ON THE DETERIORATION OF COTTON ON WET STORAGE.

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The bacterial decomposition of raw cotton on wet storage has for some considerable time been under investigation at this laboratory. A preliminary account of the progress of this investigation was published in this *Journal* about a year ago [1920]. As further material bearing on the subject has now been collected, a more detailed account appears justifiable.

It has already been pointed out that an intimate connection exists between the amount of moisture in a raw cotton and the rate of development of the bacteria present, and further, that a moisture content of 9 %, or less, is insufficient to allow bacteria to develop, so that a dry raw cotton will be protected against the action of bacteria. That this is so is shown in Table I where a sample of good Indian raw cotton with low moisture content and containing a fair number of bacteria was wetted to varying degrees.

Table I.

Percentage of moisture in cotton	Number of organisms per g. found in cotton, originally containing 1·4 millions, after 3 days at 16°
6·5	1·4 millions
7	1·5 ,,
8	2·26 ,,
9	1·4 ,,
10	124 ,,
15	499 ,,
20	1112 ,,
30	1540 ,,
50	9040 ,,

Though the above experiment explains the reason for the very large number of bacteria often met with in raw cotton, it does not indicate the source of the original, very considerable, infection of this cotton. To get further information on this point it was decided to examine raw cottons at various stages of their production, and to determine, as far as possible, the source of the infection met with. Through the kind offices of the then Director of Pro-

pellant Supplies of the Ministry of Munitions, Col. Sir Frederic Nathan, it was possible to send a request to various cotton growers in India and the United States to supply this laboratory with representative samples of raw cottons taken from ripe cotton bolls, from seed cotton which had not been ginned, from ginned, and from baled cottons, and to give a detailed description of the conditions under which the samples had been grown and harvested.

On their arrival at this laboratory the samples were examined bacteriologically and their moisture content taken. The analysis took into consideration not only the types of organisms developing on agar and gelatin plates, but also the aerobic and anaerobic cellulose-decomposing bacteria, and the thermophilic bacteria. It may perhaps be of interest to give the result of a few representative analyses, since they appear to throw some light on the source of the infection of raw cottons. The analyses of these samples are tabulated in Tables II, III and IV.

Table II. *Indian cottons (Khangaon fields).*

Sample	Agar and gelatin plates: Bacteria	Agar and gelatin plates: Moulds	Cellulose decomposing bacteria: Aerobic	Cellulose decomposing bacteria: Anaerobic	Thermophilic bacteria	Moisture %
Cotton from open ripe boll, not exposed to damp	20,000 per g. *Streptothrix chromogena* only	50,000 per g. *Aspergillus niger*	+	+	None	6·1
Seed cotton not exposed to damp	30,000 per g. *Streptothrix chromogena*	100,000 per g. *Aspergillus niger; Penicillium glaucum*	+	+	None	6·5
Cotton after ginning; sample had been picked about 14 days before ginning. During that time exposed to damp and rain though protected by tarpaulin	6,100,000 per g. *Streptothrix chromogena, Bact. herbicola aur.*	100,000 per g. *Aspergillus niger; Rhizopus niger*	+	+	+	6·1
Cotton 24 hours after pressing. Five days exposure to damp and rain before pressing	2,300,000 per g., sporing rod of Subtilis type, *Bact. herbicola aur.; B. fluorescens*	200,000 per g. *Aspergillus niger*	+	+	+	6·1

Table III. *Indian cotton from Auraiga fields.*

Cotton from ripe bolls taken immediately after picking. Some of the bolls attacked by pink boll worm. Not exposed to damp	630,000 per g., rod of Subtilis type. *S. chromogena*	80,000 per g. *P. glaucum*	+	−	None	5·6
Seed cotton just before ginning. A number of the pink boll worm found and cotton stained. Not exposed to damp	140,000 per g., rod of Subtilis type. *S. chromogena; B. herbicola aur.*	60,000 per g., *A. niger; A. oryzae* (?)	−	+	None	6·5

Table IV. *American cotton. Mississippi Delta Planting Co.*

Seed cotton exposed to little or no rain	220,000 per g., *B. herbicola aur.; S. alba;* coccus with yellow colonies	4000 per g., *Rhizopus niger*	–	–	+	8·9
Cotton after ginning; not exposed to damp	1,200,000 per g., *B. mesentericus; B. herbicola aur.; S. alba; B. fluoresc. liq.*	1000 per g., *Rhizopus niger*	+	–	+	6·9
Cotton after pressing; not exposed to damp	7,000,000 per g., *B. herbicola aur.;* coccus with yellow colonies	20,000 per g., *P. glauc.; A. glauc.*	+	+	+	7·3

It will be seen that practically all the types of micro-organisms found in these cotton samples are typical representatives of the soil flora, and there is no doubt that contamination with soil particles contributes largely to the infection of raw cottons.

Where it was possible to examine samples still in the bolls, the number of micro-organisms was always small, except in the case of the Auraiga sample, which had been attacked by the pink boll worm. Whether this pest contributed to the infection of the sample is difficult to decide. It is hoped, however, to make further inquiries into this point, when more of these samples are available.

As to the effect of damp to which some of the samples had been exposed, the evidence is contradictory. Taking the India samples alone, there certainly is a larger number of bacteria in the exposed samples than in those which had been protected. On the other hand, the American samples, which had not been damped, show quite as many bacteria as the exposed Indian cottons. In no case does the infection reach anything like the figures found in the damped Indian cottons quoted in Table I. It would appear, therefore, that at this early stage in the history of raw cottons, the wetting to which they may normally be exposed is of far less importance for the increase in number of bacteria than is the contamination with soil particles.

In addition to the typical cellulose decomposers the Streptothrix were found to be able to attack the cotton fibres. The moulds, the coccus, the bacteria of the Subtilis group and the *B. herbicola aureum* did not attack the cellulose.

The changes taking place in the cotton fibres after attack by bacteria have already been described [1920]. They were found to be so characteristic that a method, based on them, could be worked out for the quantitative determination of damaged fibres present in good cotton. An outline of this method, which may conveniently be termed the "swelling test," was given in the paper already referred to. As it has proved useful in various directions, a more detailed description of it is given below.

The swelling test is based on Balls' observation [1918] that treatment with carbon disulphide and alkali causes the cotton fibres to swell, thereby rendering the structure of the fibres visible under the microscope. On applying Balls'

treatment to a cotton fibre damaged by bacteria it was found that its structure had undergone changes, which made it readily distinguishable from a normal fibre. Microphotographs illustrating these changes were published in the short paper referred to above.

Two further microphotographs of good and damaged cotton fibres, before and after treatment with carbon disulphide and alkali, are given here. The fibres in Pl. I, fig. 1 have not been treated; of the three seen, the centre one has been damaged by bacteria, the two others not. In the second figure, where the fibres have been swelled, the third and the fourth from the top have been damaged, the others not. Comparing the two photographs it will be seen that the swelling greatly facilitates the differentiation between normal and damaged fibres.

The technique of the actual swelling of the fibres is substantially the same as that described by Balls. However, the preliminary cleaning of the fibres by boiling with 1 % NaOH, washing and neutralisation with 1 % acetic acid, has been dispensed with, except in cases of very dirty cotton samples. The fibres to be examined are now simply soaked in the mixture of alkali and carbon disulphide and left in contact with this mixture for the required time.

If a quantitative analysis has to be made to determine the damage suffered by a cotton, great care is taken to select a really representative sample for examination. This is done by very careful mixing of about 3 g. of the cotton in question.

About 0·2 g. of this uniform sample is mixed with 1·5 g. each of carbon disulphide and 15 % NaOH solution, and the flask containing the mixture is shaken well from time to time. The time during which the fibres have to be left in contact with the alkali carbon disulphide mixture varies considerably with the cotton used, and with the degree of damage, and no fixed rule can be laid down in this respect. The fibres from an Indian cotton shown in fig. 2 had been in contact with the mixture for 10 minutes, which apparently represents the minimum time advisable for this class of cottons, since the structures of the damaged fibres are not yet fully developed. Until practice has been acquired in judging when a cotton has been sufficiently treated, it is advisable to examine small test samples—a few fibres—under the microscope from time to time, say every 10 minutes.

When the degree of swelling has reached its optimum (see Figs. 2 and 3 of the earlier publication) three small samples, each of them of the size of a large pea, are taken from the swelled cotton and placed on three microscope slides. Here each sample is again carefully mixed, for instance with two ordinary pins, and about 20 fibres, taken at random, are spread out horizontally on the slides and covered with cover slips (see fig. 3). A drop of water is left to diffuse between the slip and the slide, and the preparations are ready for examination under the microscope. A magnification of × 45–50 may conveniently be used.

Ten counts are taken from each slide, the first running along the right-

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Page 410, line 9, *for* third and the fourth *read* fourth and the fifth

hand edge of the cover slip, vertically on the fibres, the following parallel with, and to the left of the first. The ten counts should cover the whole of the preparation from the right-hand edge of the cover slip to the left. The average count from each of the three slides should vary very little in well-mixed cotton samples. However, even where a considerable variation does exist, the average of these three counts has been found to give reliable figures.

In order to obtain consistent results with the swelling test, it is, of course, necessary to lay down definite rules as to when a fibre is to be regarded as damaged, and when not. It is very noticeable that the action of the bacteria is confined to small local eruptions when the fibres are first attacked, and that the damage spreads gradually over the fibre. The authors, therefore, do not count a fibre as deteriorated until it has been attacked throughout its whole length. The term fibre, as used in this connection, does not necessarily mean the whole length of a fibre, but may be any fraction of one, however small, which for some reason or other has been separated from the main part.

The swelling test has already been tried on a good many cotton samples, and has always been found both reliable and easy to carry out. In one set of experiments it was used parallel with the alkali solubility test, a test which is generally used for detecting deterioration in cottons. The results obtained appear sufficiently interesting to be given in detail.

The analyses were carried out on four cotton samples of known origin, two of which were American and two Indian. One American and one Indian sample were taken from hand-picked seed, the two others came from an American and an Indian ginning factory. Each of the four samples was wetted with its own weight of sterile distilled water and then divided into two lots, one of which was infected with a suspension of bacteria obtained from a badly deteriorated cotton, while the other was left not infected.

This infection of some of the samples was done because an earlier experiment had shown that an Indian cotton sample, straight from the bolls and containing very few cellulose decomposing bacteria, broke down very slowly unless infected with the necessary bacteria.

It soon became evident, however, that this precaution of artificially infecting the fibres had been unnecessary in this case, as even the carefully hand-picked cottons from the bolls deteriorated spontaneously at a rate scarcely slower than that of the infected samples.

After wetting and infection, the various lots of cotton were incubated at room temperature (16°) and analysed every third day.

A glance at Tables V and VI shows the striking difference in the usefulness of the two methods employed. While the swelling test (Table V) registers a steady rise in the percentage of physically damaged fibres, the alkali solubility test (Table VI) remains more or less constant, till the destruction of the cotton has reached a point, where it is affected chemically, and may be easily detected, even by the inexperienced eye.

Table V.

Sample of cotton from	Percentage of decomposed fibres found in the cotton after x days' incubation at room temperature (16°)							
	0	3	6	9	12	15	18	72
Indian bolls	0	0	4	10	16	23	19?	—
„ „ infected	0	0	4	12	22	23	30	—
American bolls	0	0	0	1	2	5?	2	54
„ „ infected... ...	0	0	0	1	2	3	3	48
Indian ginning factory	2	2	4	22	36	43	53	—
„ „ „ infected	2	3	6	24	32	39	51	—
American ginning factory ...	0	0	0	1	1	4	3	42
„ „ „ infected	0	0	0	1	1	3	2	32

Table VI.

Sample of cotton from	Percentage of decomposed fibres found in the cotton after x days' incubation at room temperature (16°) shown as percentage of solubility in alkali							
	0	3	6	9	12	15	18	72
Indian bolls	6·6	5·8	5·25	6·3	6·1	6·4	7·1	—
„ „ infected	—	5·6	5·7	5·7	6·3	5·3	7·3	—
American bolls	4·6	4·4	4·2	4·2	4·3	4·2	4·8	5·8
„ „ infected	—	4·2	4·3	4·6	4·6	5·4	4·4	6·5
Indian ginning factory	12·9?	9·6	8·55	9·09	8·5	9·09	10·3	—
„ „ „ infected ...	—	9·3	8·4	8·8	9·1	8·6	11·1	—
American ginning factory ...	5·9	5·5	5·2	5·2	4·6	4·8	5·4	6·3
„ „ „ infected	—	5·3	5·1	5·2	5·2	4·8	5·3	5·5

The alkali solubility test, therefore, does not appear to be sufficiently sensitive to be used for determining the extent to which a cotton has been physically damaged by bacteria. For this purpose the swelling test is distinctly superior, giving a clear reaction when comparatively few fibres have been destroyed.

On looking through the figures in Table V it will be noticed that the four samples of Indian cotton were much more readily attacked by bacteria than the corresponding American samples. This can scarcely be accidental, and is certainly not due to the absence of the necessary bacteria from the latter, since the infected American cottons decomposed at the same slower rate as the uninfected American samples. It seems more likely that the different behaviour of the Indian and the American cottons is due to morphological or physiological differences of the two types, caused perhaps by climatic conditions.

A further investigation of this point carried out to confirm and amplify the above results gave similar results.

Table VII gives the results of the examination of one Japanese, one Chinese, two further Indian and American and two Egyptian cottons. All the samples were wetted like the samples mentioned in Table V and left for the stated number of days at 16°. Bacteriological analysis had shown that all samples contained cellulose decomposing bacteria.

Table VII.

Exposed to damp for	Percentage of damaged fibres found in Japanese waste	Chinese waste	Indian No. 1	Indian No. 2	American No. 8	American No. 18	Egyptian silver	Egyptian scukher lap
0 days	0	0	0	0	0	0	0	0
3 „	0	0	0	0	0	0	0	0
6 „	0	3	1	0	0	0	0	0
9 „	0	3	1	2	0	0	3	1
12 „	0	10	4	2	0	0	0?	3
15 „	5	23	8	11	1	4	3	4
19 „	6	16?	13	14	2	3	4	11?
22 „	7	57	33	27	5	7	8	11

Though the two Indian and American samples of Table VII were of totally different origin to those previously tested (Table V) their rates of deterioration were almost identical, *i.e.* very slow in the case of the American, and rapid in the case of the Indian. The Egyptian cottons apparently take a middle course, being more susceptible than the American, and less so than the Indian. Of the remaining samples in Table VII the Japanese cotton compares with the American, and the Chinese with the Indian.

Judging from these experiments there is little doubt that a difference exists in the susceptibility of cottons of different origin to attack by bacteria, an observation which should be of sufficient importance from the planter's point of view to justify a careful investigation.

This investigation is now proceeding and, though little information has so far been obtained, it may be worth while mentioning, that contrary to expectations agricultural and climatic conditions do not seem to be of great importance. This will be seen from Table VIII giving the results of an analysis of two cottons grown in India from American seed.

Table VIII.

Exposed to damp for	Percentage of damaged fibres found in cotton grown in India from American seed: Gadag cotton	Akola Berai cotton
0 days	0	0
3 „	0	0
6 „	0	0
9 „	1	0
12 „	1	1
15 „	1	3?
19 „	1	1
22 „	5	4

The preliminary treatment of the two samples was the same as that used in the earlier experiments (Tables VI and VII), and both of these cottons were shown to contain sufficient cellulose decomposing bacteria to cause deterioration. Both samples are still typical American cottons from the point of view

of resistance to bacterial attack. Whatever effect, therefore, climatic and agricultural conditions may have, they are not brought to light in one generation, but must be of a cumulative nature. This and other questions are matters for further investigation.

Conclusions.

If a very large number of micro-organisms is found in a raw cotton, it is generally safe to assume that the cotton has been exposed to damp.

Smaller numbers, up to a few millions per gram, are due to a contamination of the cotton during the various stages of its preparation, including exposure on the fields.

With the help of the viscose treatment it is possible to show that changes occur in the structure of cotton fibres when attacked by bacteria. A method, based on this observation, has been worked out for the quantitative determination of the bacterial deterioration of cotton.

On applying this "swelling test" to cottons of various origins it was found that a difference exists in the susceptibility to attack by bacteria, and that Indian cottons deteriorate quicker than American samples. Samples of cotton grown in India from American seed were found to be as resistant to attack as American cottons.

References.

Balls (1918). The existence of daily layers of growth in the cell wall of cotton hairs. Author's MS.
Fleming and Thaysen (1920). *Biochem. J.* **14**, 25.

Fig. 1

Fig. 2

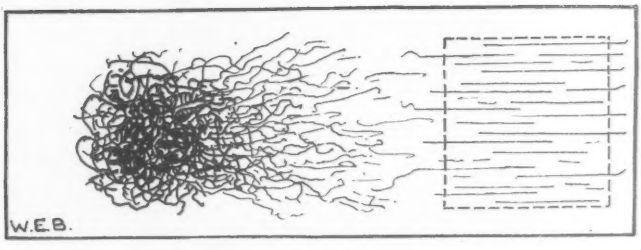

Fig. 3

XLVII. THE NATURE OF THE REDUCING SUBSTANCE IN HUMAN BLOOD.

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A rapid and convenient method of estimating sugar in blood is required for physiological investigations. With this purpose in view a research was undertaken conjointly between the physiological and biochemical departments, and a large number of estimations have been carried out by the recent method of MacLean [1919].

By this method the estimation of sugar in small quantities of blood, *e.g.* 1 cc. and even 0·2 cc. is possible. The process depends on the removal of proteins by precipitation. A measured volume of blood is first added to a 15 % solution of sodium sulphate. The mixture is then boiled, and into it whilst still hot freshly prepared "dialysed iron" is poured, which renders the protein insoluble. After filtration MacLean's solution (copper carbonate with potassium iodide and iodate) is added to a known volume and boiled. The sugar in the blood reduces the copper carbonate to cuprous oxide in presence of the iodide and iodate. The cuprous oxide is then converted into cuprous chloride by acidification, whereby a proportional amount of the liberated iodine is fixed and precipitated as cuprous iodide, and the remaining iodine is titrated with *N*/100 thiosulphate.

We decided that further work on the following lines was desirable.

(1) To ascertain whether the reducing substance estimated by the above method is entirely sugar or whether an interfering substance is present, and

(2) To discover the chemical nature of the sugar present.

The main results are summarised below. In the course of the experiments some matters bearing on technique have also been considered.

1. Technique.

(*a*) In MacLean's method the total volume is calculated by adding together the volumes of the blood, salt solution and iron, and it is assumed that there is no change in volume during precipitation. The bulky precipitate is then removed by filtration through starch-free paper and the sugar estimated in an aliquot portion of the filtrate, usually 20 cc. We found it more convenient to make up the mixture to a definite volume before filtration, *i.e.* 1·5 cc. blood to 50 cc. with distilled water. (The volume of the precipitate is negligible.) We at first cleared this by centrifuging. Subsequently we gave

up the centrifugal method for filtration through glass wool. A clear solution can easily be obtained in this way.

In order to show that no sugar was carried down in the adsorbed state by the protein precipitate, the supernatant liquor was poured off and the precipitate shaken with distilled water. On pouring off and analysing the water, no trace of sugar could be detected.

Also on adding a known weight of pure glucose to blood the reducing action of the filtrate was increased to the extent of the amount added, thus showing that none of the added sugar had been adsorbed by the protein.

(*b*) In the original method the conditions of heating the mixture of blood solution and copper salt are strictly standardised. It is recommended by MacLean that the solution should be brought to the boil in exactly 100 seconds, and that boiling should be continued for 6 minutes. In our experience the rate of initial heating has made no perceptible difference to the results. Very early in our researches we found that occasionally the results obtained were somewhat erratic which shows the importance of not relying on a single determination in pathological work. Impure distilled water, such as is supplied on the market in carboys, is one factor causing such irregularity and care should therefore be taken to ensure a pure supply of distilled water.

We have also found that the blood extract retains its reducing power without perceptible change for at least 48 hours.

2. THE NATURE OF THE REDUCING SUBSTANCES IN HUMAN BLOOD.

(*a*) Is the reducing substance in human blood entirely sugar, or are interfering substances present?

(*b*) Is the sugar entirely or largely a monosaccharide, or are di-saccharides and more complex sugars present in appreciable amount?

The results of our experiments in regard to these points are as follows:

(1) Uric acid is known to have a reducing action on copper solutions and occurs normally in the blood to the extent of 0·006 %. We therefore prepared solutions varying in strength from 0·01–0·4 % and proceeded to estimate these solutions by the method used for blood. We found that there was no reducing action under the conditions of MacLean's method.

(2) Creatinine similarly reduces copper solutions and occurs normally in the blood to the extent of 0·004 %. We prepared solutions of this strength and also 0·2 %, and proceeded in the same way as with uric acid. No reduction occurred. On adding small amounts of creatinine corresponding to the above percentages to the blood-sugar solutions, there was also no increase in reducing power.

We therefore conclude that the reducing substance is neither uric acid nor creatinine.

(3) As in the case of glucose, the reducing substance was entirely destroyed by boiling with ammonia, and was not extracted from the blood solution by means of ether.

(4) *The method of osmotic compensation.* Michaelis and Rona [1908] dialysed blood against sugar solutions of different strengths and found that when the concentration of sugar in the dialysate was the same as that found in the blood by determination, there was a state of balance. The polarimetric method was used in these experiments for estimating the sugar.

Hess and Mcguigan [1914] obtained similar results by means of *in vivo* dialysing experiments. The above authors conclude from these experiments that the sugar exists in the free state in blood and is not in the form of a colloidal complex.

We have carried out further experiments on these lines, employing the process of MacLean for estimating the sugar instead of the polarimetric method.

Small dialysers made of "viscose" were used in the experiments and the protein-free blood solution was dialysed against a solution of pure glucose of concentration slightly less than that found in the blood solution by analysis. It was found that the readjustment in concentration was such as would be expected if the reducing substance were entirely or almost entirely glucose.

We have since considered however that such experiments are by no means conclusive, because the concentrations of sugar employed are so small, that even if other substances, possessing a reducing power, were present in the blood they might not be detected by this method.

(5) *Formation of the osazone.* Several investigators, *e.g.* von Jaksch [1886], Kutz, Pickhardt [1893], and Miura [1895], have demonstrated the presence of glucose in mammalian, but not apparently human blood, by the isolation of the osazone with the characteristic microscopic appearance and melting-point.

As we intended to employ human blood throughout our experiments an attempt was made to isolate the osazone from that blood by the following procedure.

The proteins from 15 cc. of blood were precipitated by MacLean's method, and the protein-free blood solution was evaporated to dryness on the water-bath. The residue was extracted in a Soxhlet with absolute alcohol and the extract, freed from alcohol, was dissolved in a little water. The solution was then filtered and boiled for 30 minutes with 0·25 cc. of pure phenylhydrazine and glacial acetic acid. On cooling, the osazone crystallised out, and was identified by microscopical examination as glucosazone.

Pavy [1894] and many other investigators have isolated another osazone, apparently that of isomaltose, from horse blood and also from human urine.

Pavy and Siau [1901] also subsequently showed that after hydrolysing the sugar-containing solution obtained from the blood of various animals, there was a marked increase in reducing power. They calculated a factor, denoted by K, which was the percentage ratio of the reducing power determined before and after hydrolysis. This ratio varied from 66–75, according to whether the blood was obtained from the dog, cat, rabbit, or horse. Similar results were obtained in the case of human urine. They concluded that there exists in

blood and urine in addition to glucose a sugar of the maltose type, possibly isomaltose.

Cammidge [1920] has also shown that there is a hydrolysable substance in human blood, which he believes to be a dextrin. This substance increases in amount in pancreatic disease.

It seemed desirable to make a further investigation of this question, as it is likely to be of importance in clinical work, especially in following the course of certain metabolic diseases. At first we employed Pavy's original method [1894] which consists in extracting the blood with alcohol and removing the colloidal matter present in the extract with sodium sulphate. In some experiments we added a small amount of "dialysed iron" to the alcoholic extract, and dispensed with the salt altogether. The alcohol was removed by evaporation, and the extract made up to a known volume with distilled water and filtered through glass wool. The sugar was estimated in a portion of this extract by MacLean's method, and the remaining portion was hydrolysed by boiling with $N/100$ HCl for 4 hours. After neutralisation the sugar content was again determined, the results being expressed as a percentage ratio in the following way.

If a = % in sugar expressed as glucose before hydrolysis,
b = „ „ „ „ after „
then $K = \frac{a}{b} \times 100.$

We found this ratio to be very variable for different normal individuals, the range being from $K = 21$ to $K = 100$.

$K = 21{\cdot}25$	48·0	100
21·66	72·0	100
		100

When $K = 100$ there is, of course, no increase in reducing power after hydrolysis.

This method is slow and needs much manipulation; furthermore, the reducing power of a given specimen of blood was occasionally found to be much lower when this method was employed than when the usual salt-process was used.

Subsequently we simplified the process, so as to render it more convenient as a clinical method. The proteins were removed by sodium sulphate and dialysed iron as in MacLean's process, and the salt-extract was hydrolysed with HCl ($N/1$–$N/100$). In a few cases an increase in reducing power was noticed after 4 hours' hydrolysis, the ratio K varying from 36 to 58.

In many of these experiments however there was no increase in reducing power after hydrolysis for 4 hours; in fact, in some experiments there was a diminution, and even a total disappearance of the sugar originally present in the blood, pointing to destruction through the action of the acid. The amount of destruction was usually proportional to the concentration of the acid. Even when the reducing power of the blood solution was unchanged at the end of the period of 4 hours, a temporary diminution or even absence of reducing

power was sometimes noticed at the end of 1 or 2 hours, followed by a reappearance of this property after 4 hours' hydrolysis.

In the case of one person on one occasion sugar was shown to be entirely absent from the blood, as there was no reducing power before or after 4 hours' hydrolysis.

In the case of another person glucose was also absent, but the reducing power appeared after acid hydrolysis. This may mean that although there was no glucose, a hydrolysable complex sugar was present in the blood. In both these persons on every other occasion on which the blood was examined, the reducing power (before hydrolysis) was found to be normal.

There was no evidence that the presence of the sodium sulphate inhibited the hydrolytic action of the acid, as cane-sugar was easily inverted under the same conditions.

It is evident from these results, that at the present time the exact determination of the hydrolysis factor K is not available as a simple clinical method.

It was possible that the glycogen present in blood might occasionally be incompletely precipitated by the salt and "dialysed iron" and traces thus be left in the solution. This would account for the occasional increase in reduction observed after hydrolytic experiments. We therefore added a small amount of glycogen to blood and proceeded in the usual way, but found by hydrolysis that it was completely precipitated by the salting-out method.

We next attempted to ascertain the reason for the destruction of the reducing-substance present in blood during hydrolysis. In the case of $N/10$ and $N/100$ HCl the destruction is undoubtedly due to the direct action of the acid, as the amount of salt formed on neutralisation was found to have no influence at all on the reducing power of the solution. In the case of the experiments with $N/1$ acid however we were surprised to find that the presence of sodium chloride to the extent of 5 % (an approximately $N/1$ solution) had a very marked inhibitory effect on the reducing power of the blood-extract, and also on that of pure glucose and maltose.

This is illustrated by the following tabulated results.

Table I. % *of sugar.*

		In absence of NaCl	In presence of NaCl (5 %)
Blood-sugar:	(1)	0·10	0·05
	(2)	0·085	0·024
		0·243 (+added glucose)	0·199
Glucose:	(1) In water	0·102	0·087
	In 15 % Na_2SO_4	0·102	0·082
	(2) In water	0·100	0·075
	In 15 % Na_2SO_4	0·105	0·057
	(3) In 15 % Na_2SO_4	0·091	0·035
		0·231	0·139
Maltose:	(1) In water	0·071	0·032
	In 15 % Na_2SO_4	0·090	0·055
	(2) In water	0·080	0·078
	In 15 % Na_2SO_4	0·070	0·000

The above results are percentages of sugar in the original blood or sugar solution, but the actual titrations were of course carried out in much more dilute solutions (about $\frac{1}{30}$th strength). These results show that the reducing power of the blood-extract and also that of glucose and maltose is considerably diminished by the presence of sodium chloride in 5 % concentration.

But sodium sulphate has no perceptible effect, and there is thus no objection to its use as a protein-precipitant in the method of MacLean for the estimation of blood-sugar.

The difference in behaviour of sodium chloride and sulphate suggests that it is the halogen ion which exerts the specific effect.

The experiments tabulated below show that the inhibitory effect is due to a diminution in the velocity of reaction between the sugar and copper reagent, since we found by extending the period of boiling from 6 to 10 minutes that somewhat higher results were obtained.

Table II. *Glucose.*

		%	%	%
Without NaCl	6 mins. boiling	0·0070	0·0040	0·0034
Without NaCl	10 mins. boiling	—	0·0047	0·0047
With NaCl	6 mins. boiling	0·0042	0·0028	0·0028
With NaCl	10 mins. boiling	0·0066	0·0039	0·0037

It is well known that glucose forms additive compounds with halogen salts. These compounds can be obtained in a crystalline form, but are very unstable in dilute solution and are readily dissociated, and the reducing and rotatory powers of the compounds have been shown to correspond exactly to their content of glucose. This is not in agreement with the above experiments, but we have used extremely dilute solutions of sugar with a large excess of salt, so that it is still possible that the formation of the additive compound is a cause of the observed retardation. It is remarkable that this retardation is still observed even when the salt is added after the sugar solution has been raised to the boiling point, as it is unlikely that such an unstable compound would be formed under these conditions.

It is evident from the above results that in order to determine the ratio K an acid will have to be employed for hydrolysis which has no perceptible destructive effect on sugars and which forms a salt which has no retarding effect on the reducing process.

Summary.

We have carried out a number of experiments by the method of MacLean for estimating blood-sugar with the object of discovering the nature of the reducing substance or substances present.

1. Instead of filtering the bulky precipitate of protein through filter paper, it is suggested that the blood to which the sodium sulphate and "dialysed iron" have been added be made up to a known volume (1·5 cc. to 50 cc.)

with distilled water and be then filtered through glass wool. In this way a clear solution can readily be obtained.

2. Uric acid and creatinine in amounts much greater than those normally present in human blood exert no reducing action under the conditions of the above method.

3. The reducing substance is destroyed by boiling with ammonia and is insoluble in ether, as is the case with reducing sugars.

4. Phenylglucosazone with the characteristic microscopic appearance has been isolated from human blood.

5. We have confirmed the observations of Pavy and Cammidge that the reducing power of blood increases after hydrolysis with hydrochloric acid, indicating the presence of a hydrolysable substance.

6. The exact determination of the increase in reducing power after hydrolysis is complicated by at least two factors:

(*a*) The destructive effect of HCl even in low concentration upon the reducing substance of blood.

(*b*) A retarding effect on the reducing process due to the presence of the sodium chloride formed after neutralisation.

In conclusion we wish to express our grateful thanks to Professors Carlier, Ling, and Morgan for their interest and helpful advice during this investigation.

BIBLIOGRAPHY.

Cammidge (1920). *Lancet*, August 21.
Hess and Mcguigan (1914). *J. Pharm. Exp. Ther.* **6**, 45.
von Jaksch (1886). *Zeitsch. klin. Med.* **11**, 20.
Kutz (quoted by Miura).
MacLean (1919). *Biochem. J.* **13**, 135.
Michaelis and Rona (1908). *Biochem. Zeitsch.* **14**, 476.
Miura (1895). *Zeitsch. Biol.* **32**, 279.
Pavy (1894). Physiology of the Carbohydrates.
Pavy and Siau (1901). *J. Physiol.* **26**, 282.
Pickhardt (1893). *Zeitsch. physiol. Chem.* **17**, 217.

XLVIII. THE DIFFERENTIAL DIALYSIS OF THE ANTINEURITIC AND THE ANTISCORBUTIC FACTORS.

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It has been shown by Eijkman [1906] that the antineuritic factor is dialysable. Holst and Frölich [1912] also found that when fresh cabbage juice was dialysed for 3–4 days it lost the best part of its activity although all the salts did not disappear from the juice during that time. It is therefore evident that under certain conditions the antineuritic and the antiscorbutic factors will pass through a dialysis membrane. As it is now possible to calibrate the permeability of collodion membranes we set out to ascertain what degree of permeability of collodion membranes would permit the passage of these factors. Brown [1915] has shown that if air-dried collodion membranes, which are highly impermeable, are placed in solutions of alcohol and water they become permeable, that the permeability increases with the strength of the alcohol employed and that the degree of permeability of such membranes can be established by various methods based on certain properties which display a parallelism with the permeability. Utilising the above principles we have determined the permeability of the collodion membranes which permit the passage of the antineuritic factor of autolysed yeast juice and the antiscorbutic factor of lemon juice from which the citric acid has been removed.

Experimental.

The solutions were dialysed through collodion thimbles. These were prepared in the following way: a 14 % alcohol-ether solution of collodion (356A/9) supplied by the Necol Industrial Collodion Ltd was introduced into a test-tube 14 cm. long and having an internal diameter of 3 cm. The collodion was then either centrifuged or allowed to stand for about 12 hours in order to permit the bubbles of air to rise to the surface, the opening of the tube being protected by an outside tube in order to obviate the evaporation of the solvents. The tubes were inverted and the collodion was allowed to drain for 5 minutes after which time the tube with the collodion was immersed in water and the thimble was stripped off. It was then washed in water, dried for 24 hours in

the air and soaked for 24 hours in the alcohol-water solution of the requisite strength. After washing the thimbles were kept in water until required for use.

40 cc. of the active solutions were dialysed against running water. The thimbles were closed with indiarubber stoppers through which passed a glass U-tube containing mercury.

In order to ascertain the permeability of our thimbles, substances of various molecular weights were allowed to diffuse through them and the time and the degree of diffusion noted. The substances used—mostly dyes—were introduced into the thimbles which were immersed in beakers of water and the amount of the substance which diffused at various times was recorded. We have obtained the following results with thimbles soaked in 80%, 85%, 90%, 95%, 100% alcohol:

Sodium chloride had passed through all the thimbles in very considerable quantity after two hours.

Picric acid. After four days a certain amount of the substance had passed through the 80 % membrane. As in the case of the following substances the amount which diffused in that time increased with the strength of the alcohol employed in the treatment of the thimbles, the 100 % membrane showing the greatest permeability.

Potassium oxalate. None of the salt had passed through the 80 % membrane in four days; a certain amount diffused through the 85 % membrane.

Bismarck brown. None of the substance passed through the 80 % and the 85 % membranes; a certain amount diffused through the 90 % membrane.

Methylene blue. None of the substance passed through the 80 % and 85 % membranes; a trace diffused through the 90 % membrane.

Neutral red. None of the substance passed through the 80 %, 85 % and 90 % membranes; a certain amount of it diffused through the 95 % membrane.

Safranine behaved almost like neutral red.

Dextrin. None of the substance passed through the 80 %, 85 %, 90 % membranes; a trace diffused through the 95 % and a little more through the 100 % membranes.

Litmus. None of the substance passed through the 80 %, 85 %, 90 % and the 95 % membranes, some through the 100 % membrane.

Congo red did not pass through any of the above membranes in four days.

Autolysed yeast was used as the source for the antineuritic factor. The yeast after being washed free from wort was pressed out and placed in a large flask plugged with cotton wool in the hot room at 37° for a few days. The autolysed mass was filtered through a large Buchner funnel when sufficiently liquified and the brown filtrate thus obtained was dialysed for four days as described above and fed to rats which subsisted on a diet deficient in the antineuritic factor and which manifested signs of decline. Resumption of growth showed that the accessory factor had not totally diffused in that time. Failure to induce growth, on the other hand, showed that the active

substance had entirely passed through the membrane during the four days of dialysis.

As the source for the antiscorbutic factor lemon juice was employed which had previously been treated with an excess of calcium carbonate and filtered. This antiscorbutic solution, which was dialysed for three days, was tested on guinea pigs kept on a scorbutic diet of oats, bran and autoclaved milk. The administration of the doses was carried out about fourteen days after the animals had been put on the deficient diet, and in all the cases where the antiscorbutic factor diffused scurvy developed, in the other cases the onset of the disease was prevented.

A sample of the original material was always kept in a glass flask in the dialysing tank under the same conditions as the dialysing thimbles and was utilised for control purposes.

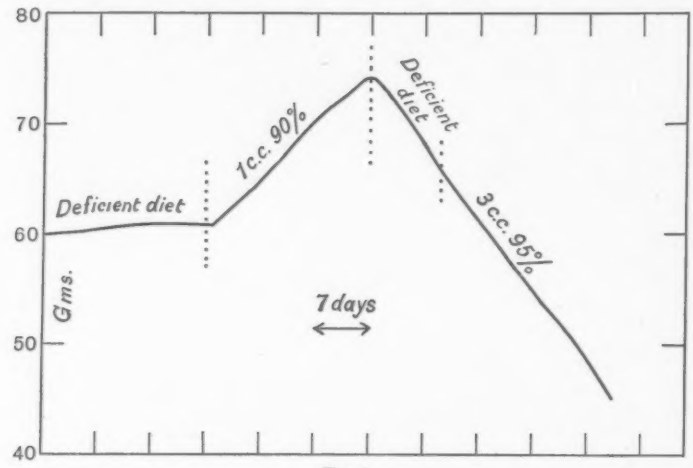

Fig. 1

Membranes of various permeabilities were tried until the one which allowed the free diffusion of the factors was obtained. In the case of both the antineuritic and the antiscorbutic factors it was found that the accessory factors would not pass through a 90 % membrane whilst they diffused entirely through a 95 % membrane in the time mentioned.

It will be seen from Fig. 1, which represents the growth curve of a rat kept on a diet deficient in the antineuritic factor, that an equivalent of 1 cc. of autolysed yeast dialysed through a 90 % membrane induced growth in the animal. As 1 cc. of autolysed yeast juice is approximately the minimum dose which is capable of inducing growth in a rat fed on a diet deficient in the antineuritic factor, one may conclude that very little of the factor diffused through this membrane in four days. The same animal was then placed again on the

deficient diet, and as was to be expected the rat declined in weight. The addition of 3 cc. of autolysed juice dialysed through a 95 % membrane for four days failed to promote growth, thus showing that the antineuritic factor had gone through entirely in this time. Other experiments have confirmed this observation: in all cases the factor failed to diffuse in any appreciable extent through a 90 % membrane but did so entirely through a 95 % membrane.

Similarly it will be seen from Fig. 2 that 7 cc. of decitrated lemon juice dialysed for three days through a 95 % membrane failed to prevent or even delay the onset of scurvy in a guinea-pig, whilst 3 cc. of the juice which had been dialysed through a 90 % thimble prevented the onset of the disease. The antiscorbutic factor therefore behaved much in the same way as the antineuritic factor. In this case also various experiments confirmed this result.

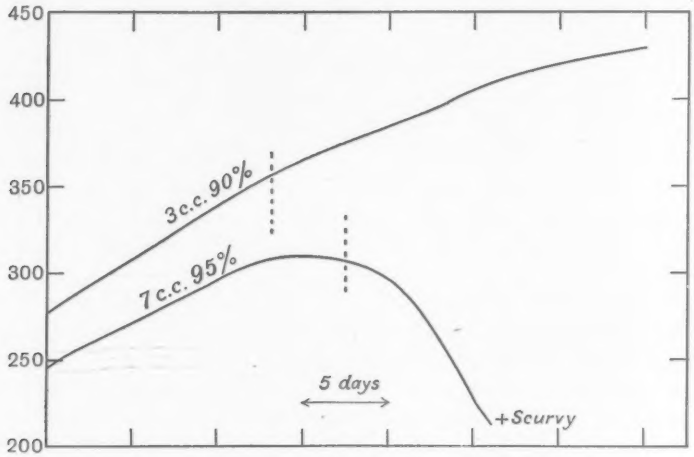

Fig. 2

A number of experiments with 35 %, 50 % and 70 % membranes showed that they were not permeable to the above accessory factors. These principles however passed freely through a 100 % thimble.

It is thus seen that the antineuritic factor in autolysed yeast juice and the antiscorbutic factor in decitrated lemon juice diffuse through membranes of such permeability as permit the passage of substances of such molecular dimensions as methylene blue, neutral red and safranine. From this one may conclude that the dimensions of the molecules of these factors or the molecules with which they may possibly be associated are of the order of that of a semi-colloid. There is, however, no evidence whether these molecules are simple or associated. It must also be pointed out that the above experiments were made without the application of pressure and only one source for each of the accessory factors was used. It would be of interest to investigate whether

factors such as hydrogen ion concentration, which influence the degree of dispersion, also influence the diffusibility of the antineuritic and antiscorbutic principles.

SUMMARY.

The antineuritic and the antiscorbutic accessory factors diffuse through a collodion membrane of such permeability as permits the passage of substances like methylene blue, neutral red and safranine. Membranes of lower permeability were found not to allow the diffusion of these factors. It is suggested that the active molecules whether simple or associated may be of a semi-colloid nature.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

REFERENCES.

Brown (1915). *Biochem. J.* **9**, 591.
Eijkman (1906). *Arch. Hyg.* **58**, 150.
Holst and Frölich (1912). *Zeitsch. Hyg.* **72**. 1.

XLIX. THE RELATION OF THE FAT-SOLUBLE FACTOR TO RICKETS AND GROWTH IN PIGS.

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Clinical and experimental observations tend to show that diet plays an important part in the etiology of rickets and much of the evidence obtained by the clinician goes further to demonstrate that the results obtained with certain oils and fats in the prophylactic and the curative treatment of the disease is quite marked. The recent experimental work of Mellanby [1918 and 1919] throws a very interesting light on the therapeutic value of fats and oils. Mellanby has shown that by keeping puppies on a certain basal diet, definite rickets could be induced in these animals and that the disease could be prevented or cured by the addition of certain substances, mostly oils and fats, to the basal diet. Of the substances which were capable of producing this beneficial effect the majority were found to be identical with those which contain the fat-soluble A factor. This important observation suggested the possibility that the fat-soluble factor or another accessory factor closely associated with it was concerned in the prophylaxis of rickets. However, investigations carried out clinically and experimentally in order to test this theory of the etiology of rickets have yielded results from which no definite conclusions can yet be drawn. Hess and Unger [1920] from observations made on groups of infants receiving diets some of which were rich, others deficient in the fat-soluble factor, could not obtain any definite evidence that the rickets which developed in some of their patients could be traced to an accessory factor deficiency. Harden and Zilva [1919] kept young monkeys on a diet deficient in the fat-soluble factor for several months. The animals declined, but no rickets developed. Mackay [1921] fed kittens on a diet deficient in this factor, but otherwise theoretically adequate, with the result that the animals ceased growing. No evidence of rickets however could be established at the post mortem examination. McCollum, Simmonds, Parsons, Shipley and Park [1921] recently analysed a series of diets on which rats developed rickets and although the

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results pointed to the possibility that a deficiency of the fat-soluble factor or calcium might be responsible for the production of the disease, the authors considered that with the experimental evidence so far at their disposal they were only justified in concluding that faulty nutrition was the cause of the observed rachitic changes.

We are at present engaged in studying various dietetic problems including the etiology of rickets in the pig. The pig is a very suitable animal for such a purpose as it is susceptible to rickets, is omnivorous and can be reared away from its mother on experimental diets.

The cause of rickets in the pig has often been attributed by agriculturists to a dietetic deficiency—calcium and phosphorus being the suspected elements. Thus one finds statements in various text-books on scientific feeding that pigs fed on potatoes, whey, maize and cereals develop rickets. If on the other hand such diets are supplemented by clover or meadow hay the disease does not occur. Although calcium is usually considered to be the limiting factor it will be noticed that the diets which are alleged to produce rickets in the pig are at the same time deficient in the fat-soluble factor, whilst clover and meadow hay besides supplying calcium also form a source for this accessory factor.

Herter [1898] on the assumption that fat deficiency might be responsible for the production of rickets placed young pigs two months old on a diet of skimmed cow's milk. The animals deteriorated after several months but in the author's opinion rickets did not develop. It is, however, of interest to note that Herter found that phosphates were imperfectly absorbed when this diet low in fat was used. The diets were of course also low in the fat-soluble factor.

Our primary object was to find out whether pigs, brought up from birth on a diet rigorously restricted in the fat-soluble factor only, would develop rickets with the regularity with which animals, which are susceptible to scurvy, develop this disease on a scorbutic diet. Owing to the exacting attention that such experiments require we could only deal at first with a limited number of animals. The results obtained concerning the original object in view were not definite, but other observations of interest were made which will be discussed in this communication.

Experimental.

The experiments were carried out at the Reading University College Farm, Shinfield. Four animals, *A*, *B*, *C* and *D*, which were divided into two groups, were employed in this experiment. Group I, consisting of *A* and *B*, were placed on a diet containing the fat-soluble factor, whilst Group II, consisting of *C* and *D*, were kept on a diet rigorously restricted in that factor. The animals, which weighed three pounds each at the commencement of the experiment, were young Berkshire boars farrowed on August 13th out of "Whitley Sensation" by second prize boar "Swinton Cogniac." On August 17th they were placed on the experimental diets. During the first three days a mixture resembling

in composition sow's colostrum was given to the animals. The diets were of the following composition:

Group I.		*Group II.*	
Extracted dried milk	7·75 g.	Extracted dried milk	7·75 g.
Cream (cow's milk)	20·2 „	Autoclaved olive oil	9·5 „
Purified caseinogen	12·6 „	Purified caseinogen	13·0 „
Salt mixture	0·28 „	Salt mixture	0·36 „

The above was made up to 100 cc.

On August 20th the above diets were changed to mixtures approximating in composition to sow's milk, which were made up according to the following formulae:

Group I.		*Group II.*	
Fresh cow's milk	60 cc.	Extracted dried milk	6·7 g.
Crude caseinogen	5·0 g.	Purified caseinogen	4·9 „
Cream (cow's milk)	5·0 „	Autoclaved olive oil	4·6 „
Salt mixture	0·66 „	Salt mixture	0·63 „

The above was made up to 100 cc.

To insure the solution of the caseinogen 5 % sodium bicarbonate (20 cc. per 100 g. of caseinogen) was used.

Daily doses of decitrated lemon juice and "marmite" were administered to supply the antiscorbutic and the antineuritic requirements of the animals.

The dried separated milk was extracted with light petroleum for two to three days.

The cream was freshly separated and was obtained daily from the Reading College Dairy. Its average fat content, as determined by the Gerber method, was 42 %.

The caseinogen was rendered free from detectable traces of the fat-soluble factor by being heated for 24 hours in shallow layers at 120° C. and being subsequently extracted for one day with 90 % alcohol and for two days with light petroleum. The olive oil was autoclaved for ½ hour at a pressure of two atmospheres. The salt mixture was of the same composition as that employed by McCollum and his collaborators.

As will be seen, the chief difference between the diets in the two groups was in the content of the fat-soluble factor. In Group II the active cream was replaced by the inactive olive oil.

At the commencement of the experiment the little pigs were fed by bottle every two hours day and night. After August 23rd the intervals were extended to three hours. By the end of August the animals received six meals a day, which were further reduced by September 15th to five meals, with a night interval of six hours. The diets were given *ad lib.* and the quantities consumed were recorded.

At first the intake of food and the growth of the two groups were approximately the same. After a few days, however, the animals in Group II commenced consuming less food than the animals in Group I and at the same

time ceased growing, whilst the control animals continued to gain in weight. See Photograph 2, Plate II.

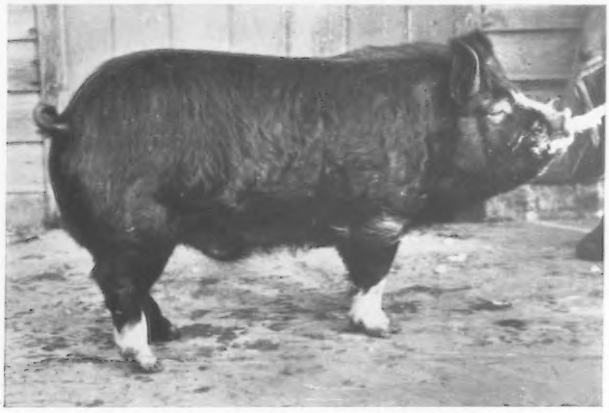
On September 14th pig *D* died during the night. A skiagraph was taken soon after death, but no abnormalities could be discerned. At the post mortem examination very pronounced "beading" at the costo-chondral junctions of the ribs was observed. Photograph 1, Plate II, shows the appearance of the costo-chondral junctions of the ribs of this animal. No other abnormal changes were established. The analysis of one of the long bones gave the following figures: moisture, 20·1 %; CaO, 29·3 % of the dry weight. We have so far been unable to obtain a really normal animal of the same age for comparative purposes. Histological preparations of the ribs, which will be discussed later, were kindly made for us by Miss F. M. Tozer, to whom we take this opportunity of expressing our best thanks.

In order to save the other declining animal in this group, cream was added to its diet in the same proportion as that in the diet of Group I. It was intended to supply a small amount of the fat-soluble factor sufficient to stop further decline. The animal soon resumed growth and the cream was accordingly discontinued on September 27th. From September 14th this animal received, besides the artificial milk mixture, an addendum of a basal mixture made up of 150 parts of starch, 40 parts of purified caseinogen and 10 parts of the salt mixture. After the cream was discontinued crude caseinogen was employed in this mixture instead of the purified substance in order to supply a further very limited amount of the fat-soluble factor. An average of about 250 g. of this mixture was consumed by the animal per day. From October 16th purified caseinogen was again employed, and on October 26th the artificial milk was replaced by a mixture consisting of olive oil, extracted dried milk, basal mixture and salt mixture. The olive oil and the caseinogen were discontinued on November 22nd. At this period the animal was consuming about 2 lb. of starch and $1\frac{3}{4}$ lb. of extracted fat-free dried milk per day. Towards the end of the experiment, owing to a temporary shortage of extracted dried milk, a mixture of starch and purified caseinogen was again resorted to.

As will be seen from Fig. 1, which represents the weight curves of the animals, pig *C*, in spite of the restricted diet, kept on growing after the resumption of growth produced by the administration of the cream even better than the animals in the control group. No symptoms of rickets were observed during the experimental period. A skiagraph was taken on November 9th and a normal picture was obtained. The animal was slaughtered on January 19th. At the post mortem examination the following was found: Skin—good and clean. Thyroid glands—more connective tissue than in the control animal. Femur decidedly thicker and distinct enlargement of all bones at knee joint. Tarsal bones thicker than in control. Junctions between tarsals and metatarsals also thicker than in control. Spleen—a little heavier than in control. Liver—superficial foci of inflammation; weight of liver, $2\frac{1}{4}$ lb. More fat was found in this animal than in the control. Kidney normal. Adrenals

Photograph 1. Ribs from *D*.

Photograph 2. *A* and *C* 28 days old.



Photograph 3. *C* before being slaughtered.

Photograph 4. Litter from Sow Lot I.

Photograph 5. Litter from Sow Lot I.

Body weight in lbs.
140
130
120
110
100
90
80
70
60
50
40
30
20
10
Starch given
Toppings and whey started
Starch mixture given
Wheat germ started
Toppings and whey started
Cream given
Crude casein started
Extracted casein given
Dried milk and starch
Died
D (no cream)
Starch given
A
B
C
4 months
Daily intake of dry matter in lbs
1·0
0·5
0
B's intake
A's intake
C's intake
D's intake

Fig. 1

normal. Ribs—angle of junction between bones and cartilage at costo-chondral junction markedly greater than in the control. In attempting to cut with a knife the same resistance was met as in the case of the control. The cartilage at the costo-chondral junctions strikingly thinner than the bony part as compared with the control animal. Longitudinal section at costo-chondral junction is decidedly wider than in the control and the line of active cartilage is also a little wider. Heart and lungs quite normal. Weight of leaf fat, 1 lb. 14½ oz. The analysis of one of the long bones gave the following figures: moisture, 14·7 %; CaO, 37·6 % of the dry weight. Photograph 3, Plate II, is a picture of pig *C* before being slaughtered.

In the control group pigs *A* and *B* commenced showing weakness in their legs, animal *A* in the hind and animal *B* in the front limbs and afterwards also in the hind legs. The wooden floor was at this stage replaced by bricks and the animals somewhat improved soon after the change. This affection of the limbs was definitely established to be non-rachitic. On September 13th an addendum of basal mixture and wheat germ was made to both diets. From September 20th lucerne was given to pig *B* and from October 1st about 15 g. of crude cod-liver oil was administered to pig *A*. On October 6th the milk was discontinued in both cases, pig *A* receiving basal mixture, wheat germ, toppings and whey; pig *B* receiving crude caseinogen, toppings and whey. From November 22nd the diet of pig *A* consisted of toppings, whey and cod-liver oil, which was supplemented by maize gluten on December 23rd. The diet of pig *B* was supplemented by maize gluten on December 15th. Pig *B* was killed on January 19th and pig *A* on February 17th. At the post mortem examination no rickets could be established in either case. A piece of wire was found to have penetrated the stomach of pig *A* and to have caused local inflammation which most probably was responsible for the ill-health of the animal during the last few weeks of the experiment. The analyses of the long bones gave the following figures: pig *A*, moisture 16·8 %, CaO 35·5 % of dry weight.

On three occasions during the early part of the experiment the weights of our experimental animals were compared with those of the little pigs of the same litter which remained with the mother. On September 8th the pigs in the litter weighed 16½, 15, 13, 14½, 14¼ lb. whilst pig *A* weighed 14 lb., pig *B* 12¾ lb., pig *C* 8 lb. and pig *D* 9¾ lb. On September 24th the pigs in the litter weighed 21¾, 23¾, 26¼, 18½ lb., and pig *A* weighed 25½, pig *B* 22¾, pig *C* 12¼ lb. On November 26th the average weight of the litter pigs was 54·7 lb., whilst pig *A* weighed 65¼, *B* 72¼ and *C* 72 lb. It is therefore evident that the pigs brought up artificially gained more weight than the animals which remained with the mother.

The ribs of pigs *A*, *C* and *D* were examined histologically and the observations may be summed up as follows:

(1) A moderate increase in the number of proliferating and hypertrophic cartilage cells was found only in pig *C*.

(2) A deep penetration of blood vessels from the bone marrow into the cartilage was observed in all the three cases.

(3) The presence of fibro-cellular tissue instead of normal marrow was found in all cases. It was more pronounced in the case of pig *C*.

(4) The irregularity of the line at the costo-chondral junction was also recorded. This was most marked in the case of pig *D*.

The back and leaf (perinephritic) fats from pigs *A*, *B* and *C* were tested by our standardised technique for the presence of the fat-soluble factor on rats, and as will be seen from Fig. 2 which represents the weight curves of these rats, the fats derived from animals fed on a diet containing this factor were found to be more or less active; the reverse was the case with the fats from the animal fed on a deficient diet. There was one discrepancy in the case of the leaf fat of pig *B* which appears to have been due to an irregularity in the technique; unfortunately we did not have enough of this sample to repeat the test. These observations confirm the results we [1920] have previously obtained with pigs fed on diets of varying contents of the fat-soluble factor.

No marked difference was found in the water, connective tissue and nitrogen contents of the two fats. The refractive indices, the Polenske, iodine and potash absorption values were also almost identical.

The results of our experiments offer no definite information on the relation of the fat-soluble factor to the etiology of rickets. We are however of the opinion that if the deficiency of this factor alone bore the same relation to the etiology of rickets as that of the antiscorbutic and antineuritic factors to the etiology of scurvy and beri-beri, we should have obtained a better differentiation in the rachitic condition in the two groups, in spite of the limited number of animals employed. As it is, we find it impossible with the results so far at our disposal to explain the decided, although slight, rachitic changes observed histologically in our normally-fed animals.

The above experiments, however, bring into prominence the following points:

(*a*) That when pigs are "off their feet" it does not necessarily imply that it is due to rickets. The affection of the limbs of pigs *A* and *B* was decidedly not of a rachitic nature.

(*b*) That the requirements of the pig for the fat-soluble factor are of a low order. This has already been pointed out by us [1920] in a previous communication. In this investigation the comparatively small quantity of cream and crude caseinogen consumed by pig *C* was sufficient to act as a source of the fat-soluble factor for the animal during about four months.

(*c*) That the fat-soluble factor promotes growth in the pig. This was demonstrated by the resumption of growth of pig *C* on the addition of cream to the restricted diet. It was further confirmed by the following experiment, which is a continuation of that described in the previous communication already referred to and in which we kept pigs on diets consisting of toppings

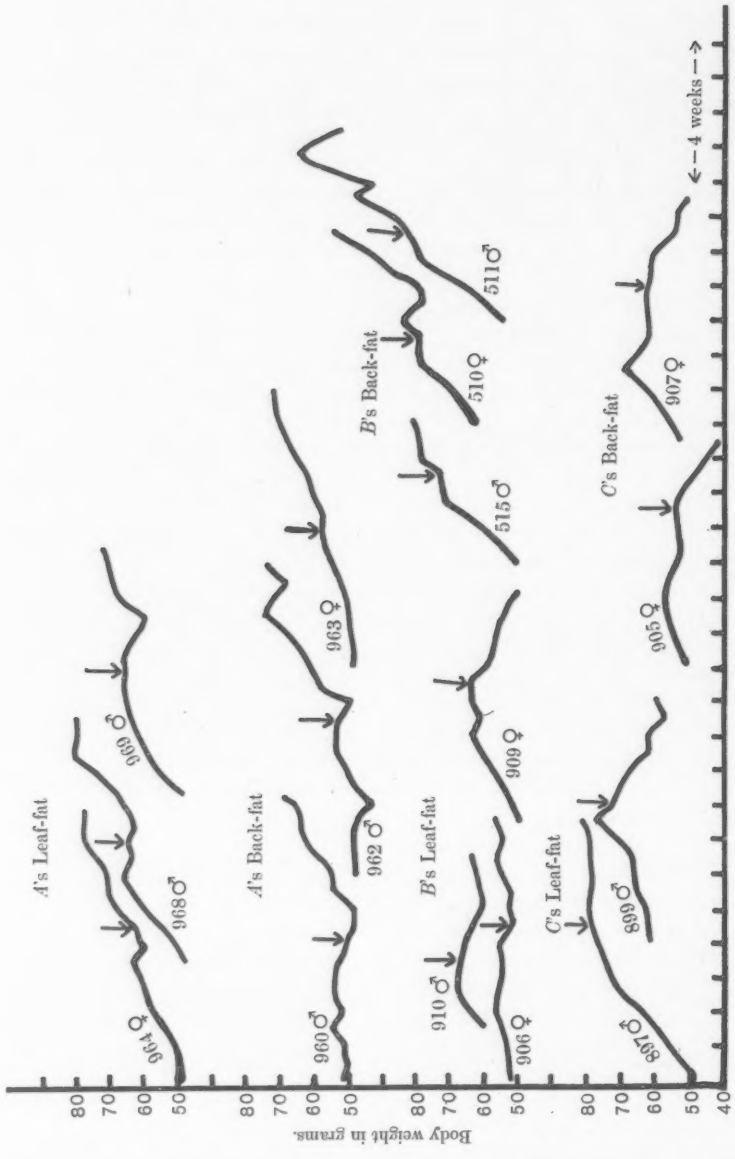

A's Leaf-fat
A's Back-fat
B's Leaf-fat
B's Back-fat
C's Leaf-fat
C's Back-fat
Body weight in grams.
← 4 weeks →
968♂
969♂
960♂
962♂
963♀
910♂
906♀
909♀
515♂
510♀
511♂
897♂
899♂
905♀
907♀
80
70
60
50
40

Fig. 2

and synthetic whey (Lot I) and toppings and ordinary whey (Lot II). Both these diets were highly deficient in the fat-soluble factor, the diet of Lot I being the more restricted. In the early stages of the experiments these animals showed a considerable growth which eventually slackened off. On September 23rd the sows belonging to Groups I and II had their restricted diets supplemented by an addendum of the fat-soluble factor. At this time the animal in Lot I had practically ceased growing, whilst the animal in Lot II was growing at a rate much below the normal. It was decided to administer the fat-soluble factor to the sow in Lot I in the form of crude cod-liver oil which was found to be very active in the case of rats and to the sow in Lot II in the form of lucerne. Lot I was given at first $\frac{1}{4}$ oz. of cod-liver oil per day; this was after five days increased to $\frac{1}{2}$ oz., and after 42 days this was again increased to 1 oz. per day for 20 days. The animal consumed the oil with great alacrity and resumed growth immediately after the commencement of the treatment. Lot II was started at first on $\frac{1}{2}$ lb. of lucerne per day; this after five days was increased to 1 lb., and after two weeks to 4 lb. per day. On December 3rd both sows were taken to the boar and became pregnant. They were then placed again on their original restricted diets. Fig. 3 represents the weight curves of these animals as well as that of the sow belonging to Lot III, which was fed all the time on toppings, whey and grass, a diet rich in the fat-soluble factor. On March 30th the sow in Lot I gave birth to eight pigs all of which were born dead or died very soon after birth.

One of the young lived for five hours and on post mortem examination was found to be normal except for a slight general oedema. Of the remaining seven pigs four showed a marked abnormality in the development of the hind limbs to a varying degree. This malformation was particularly pronounced in two cases (see Photographs 4 and 5, Plate II) in which the hind limbs were represented by thin tail-like appendages.

Oedema was generally present in all the eight bodies to a varying degree; four cases showing very marked ascites, hydropericardium and hydrocele, but there was no association between the severity of the oedema and the malformation of the limbs. The large hydroceles in these cases may be seen from the photographs. In nearly all cases there was more or less severe hydronephrosis.

The weights of the eight young were respectively 950, 1100, 1250, 900, 950, 1050, 1100 and 1350 g.

Analyses of the bones of three of the pigs were made, but their value is limited by the fact that only one analysis from a normal pig of the same age was available.

	Pigs from litter Lot I.			Normal control
	1	2	3	
	g.	g.	g.	g.
Weight of humerus	5·2	5·68	4·27	4·5
% moisture	41·6	46·3	44·5	40·9
% CaO on dry weight	34·45	33·71	32·80	37·24

Body weight in lbs.
350
300
250
200
150
100
50
Sow Loc III. Toppings. Whey and Grass
Sow Loc II. Toppings and Whey
Sow Loc I. Toppings and Synthetic Whey
Cod liver oil begun
Served by boar 1540
To boar
Lucerne begun
Served by boar 1540
Cod liver oil discontinued
3 months

Fig. 3

We are not at present in a position to say whether this abnormal litter is to be ascribed to the drastically restricted diet which the mother had received, but further experiments are in progress.

The sow in Lot II farrowed down nine pigs on April 8th of which only one died.

In conclusion we wish to express our indebtedness to Mrs John Golding for the indefatigable help she rendered us during this inquiry.

The expenses of this research were defrayed from a grant made by the Medical Research Council, to whom our thanks are due.

Summary.

No definite rickets was induced in sucking-pigs fed from birth on a diet rigorously restricted in the fat-soluble factor.

The addition of the fat-soluble factor in the form of cream, cod-liver oil, and lucerne to a deficient diet stimulated growth in pigs declining in weight.

REFERENCES.

Drummond, Golding, Zilva and Coward (1920). *Biochem. J.* **14**, 742.
Harden and Zilva (1919). *Lancet*, 780.
Herter (1898). *J. Exp. Med.* **3**, 293.
Hess and Unger (1920). *J. Amer. Med. Assoc.* **74**, 217.
Mackay (1921). *Biochem. J.* **15**, 19.
McCollum, Simmonds, Parsons, Shipley and Park (1921). *J. Biol. Chem.* **45**, 343.
Mellanby (1918). *J. Physiol.* **52**, *Proc.* xi.
—— (1919). *Lancet*, 407.

L. THE SYNTHESIS OF VITAMIN B BY YEASTS (PRELIMINARY NOTE).

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(*Received May 5th, 1921.*)

Experiments on this subject have been in progress in this laboratory for a considerable time with the twofold object of ascertaining whether yeast grown on a medium devoid of vitamin B is able to produce this substance and whether different species of yeast all produce this vitamin. Our experiments are not complete but in view of the recent publication by Nelson, Fulmer and Cessna [1921] we think it desirable to make a brief statement of the results so far attained.

The method used consists in growing the yeast under examination on a synthetic medium containing ammonium phosphate and chloride as sources of nitrogen, together with the necessary mineral salts and cane-sugar. The cane-sugar was fractionally precipitated by alcohol from aqueous solution, and the solution of the dried purified material then shaken three times with fuller's earth to remove any possible trace of vitamin B.

The specimen of *S. cerivisiae* used had been isolated from a sample of baker's yeast, and *S. ellipsoideus* was also examined.

S. cerivisiae grew very slowly and imperfectly in the medium, whereas *S. ellipsoideus* grew much more rapidly and gave a larger yield.

The yeasts were centrifuged out of the medium, washed three times with distilled water, pressed and dried in the air. A parallel culture of *S. ellipsoideus* on unhopped brewer's-wort was made and the yeast crop treated in a similar manner.

The dried yeasts were then compared as regards their curative effect on pigeons suffering from avian polyneuritis as a result of a diet of polished rice, and showing retracted neck. Removal of this symptom for three days was regarded as a "standard" cure.

The following is a brief statement of the results.

Material	Dose g.	Effect
Brewer's yeast	1	Cure
„ „	0·5	„
S. cerivisiae from synthetic medium	1	Temporary cure, but recurrence after 48 hours
S. ellipsoideus from wort	1	Cure
„ „ „	0·5	„
„ „ synthetic medium	1	„ (lasting 4 days)
„ „ „ „	1	„ („ 6 „)
„ „ „ „	0·5	Temporary cure, but recurrence after 48 hours

It appears therefore that *S. ellipsoideus* produces vitamin B and that the yeasts grown on the synthetic medium contain vitamin B, but not in so large a proportion as those grown on wort. Further experiments are in progress.

The expenses of this research were in part defrayed from a grant for which we are indebted to the Medical Research Council.

REFERENCE.

Nelson, Fulmer and Cessna (1921). *J. Biol. Chem.* **46**, 77.

LI. THE ACTION OF SALTS AND NON-ELECTROLYTES UPON BUFFER SOLUTIONS AND AMPHOTERIC ELECTROLYTES AND THE RELATION OF THESE EFFECTS TO THE PERMEABILITY OF THE CELL.

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(*Received March 15, 1921.*)

Introduction.

The presence of buffer solutions in living protoplasm is generally assumed by physiologists, although it is only in a very few instances that direct chemical evidence of their presence has been obtained. The mixture of phosphates and carbonates which exists in the blood is well known and has been the subject of much examination, both theoretical and experimental, and it has recently been shown [Hempel, 1917] that certain vegetable saps contain buffer mixtures of a different type—mixtures of the acid and neutral salts of organic acids. The existence of buffer mixtures as a normal constituent of the living protoplasm of the cell is however a hypothesis which it is impossible at present to submit to experimental verification. It is nevertheless supported by much indirect evidence and gives a simple and entirely satisfactory explanation of the way in which the organism is able to maintain the reaction of its protoplasm very near to absolute neutrality in spite of the continuous production of carbon dioxide and other acidic substances.

It is perhaps in consequence of the great importance of the neutralising action of buffer solutions that attention has been focussed upon this property to the exclusion of others which may have an equal physiological significance. The direct action of salts upon the proteins of the protoplasm has been the subject of much study, but little attention has been paid to the indirect action which they may exert by modifying the reaction of the buffer solutions which bathe the protoplasm, although this effect is well known to take place. The whole trend of modern research is however to lay stress upon the importance of the reaction of the medium in which physiological processes occur, and the modifications of reaction which buffer mixtures undergo, not only by the addition of salts, but also by the addition of non-electrolytes and by changes

of concentration and temperature, cannot fail to be of importance. Most of these changes can be deduced very simply from the theory of buffer solutions and they can also be demonstrated by experiment. The theory of buffer solutions will therefore be very briefly outlined here in order to emphasise these neglected elements, and the results will be given of some series of experiments which have been undertaken to confirm the theoretical deductions and to obtain some estimate of the order of magnitude of the effects upon which it is desired to lay stress. Finally an attempt will be made to discuss the bearing of these results upon the theory of certain physiological processes.

Theory of buffer solutions.

Although the complete series of equations which determines equilibrium in a buffer solution is usually complex, since a number of ions and undissociated molecules may be present, it is generally possible to neglect a number of these equations without introducing any considerable error and to regard the properties of the mixture as due to the balanced dissociation of two substances—weak acid and salt—having a common ion. The equations which determine the properties of the mixture may then be generalised in the form

$$H_2X \rightleftarrows H^{\cdot} + HX'$$
$$MHX \rightleftarrows M^{\cdot} + HX'$$

in which case the dissociation and hydrolysis of HX' are neglected as well as any formation of undissociated M_2X.

Henderson [1908] has worked out the theory of buffer solutions in this form and has applied it to mixtures of carbonates and phosphates. He points out that H_2X, being a weak acid, is very slightly dissociated in the presence of its salts, and that MHX may therefore be regarded as the sole source of HX' ions. The concentration of hydrogen ions is therefore determined by the equations

$$[H^{\cdot}] = k\frac{[H_2X]}{[HX']} = k\frac{[H_2X]}{\alpha[MHX]}$$

where k is the first dissociation constant of the acid H_2X and α is the degree of dissociation of the salt MHX.

The concentration of hydrogen ions is therefore a function of

(1) the concentration of H_2X;
(2) the concentration of MHX;
(3) the value of α;
(4) the value of k.

The effects of changes in the relative concentrations of acid and salt has been very fully dealt with by Henderson and others. It is proposed here to enquire into the effect of changes in the other factors which determine acidity —the degree of dissociation of the salt, and the dissociation constant of the acid—factors which up to the present have been almost completely ignored. Since $[H^{\cdot}]$ varies inversely as α, the acidity of the solution will vary with any

change in the degree of dissociation of the more alkaline constituent of the buffer mixture, whether this change be due to an alteration in the active mass of salt present or to any change of external conditions which affects its dissociation constant. In this latter case the value of k, the dissociation constant of the acid, will also tend to change—probably in the same direction. It is possible however that in certain cases, which will be touched upon later, the dissociation constants of acid and salt may vary in opposite directions, in which case the effect of the variations will be greatly increased.

It is possible from the equation

$$[H^{\cdot}] = k \frac{[H_2X]}{\alpha[MHX]}$$

to predict the general effect of many of the changes which can be produced in a given buffer mixture. Unfortunately this is not always the case. Conditions are frequently so complex that to unravel their effects becomes a very formidable task, while in other cases the data necessary for calculation are altogether lacking. It will be worth while, however, before proceeding to detail experimental results, to enumerate the more important conditions affecting the values of k and α and hence of $[H^{\cdot}]$, and to estimate as far as possible the bearing of theory upon them.

(1) *Change of concentration.* Decrease of concentration will increase the value of α and will leave k untouched, consequently the acidity of a buffer mixture will decrease with dilution. This effect is small but it seems doubtful whether it is so entirely negligible as has been assumed by Henderson and by Bayliss [1915]. The order of magnitude of the effect will be seen in the experiments on phosphate and acetate mixtures given below.

(2) *Addition of neutral salts.* The addition of a salt MX' to the mixture MHX, H_2X will increase the concentration of the cations and thus reduce the dissociation of MHX. Increase of acidity will therefore follow. If the salt added has a different cation, M', the conditions are rather more complicated. If however M' is univalent the mixture $M'HX$, MHX will have properties very similar to those of the simple salt. A bivalent cation is in a different category; it produces a far greater increase of acidity. This is due primarily to the formation of the less dissociated salt $M'(HX)_2$, but other salts may also be formed as *e.g.* $M'X$. A comparison of the effect of magnesium chloride in phosphate and acetate mixtures will serve to show that the possibilities which polybasic acids afford for the formation of compounds of this type is not to be ignored.

The effect of the anion has hitherto been disregarded. When the salt $M'X'$ is the salt of a strong monobasic acid both $M'X'$ and HX' may be regarded as completely dissociated and the anion X' will be without effect. Where however HX' is not completely dissociated, a second mixture of acid and salt having buffer properties will be formed and the net result may be either to increase or decrease the value of $[H^{\cdot}]$. A particularly interesting case is that of the polybasic organic acids. Acids of this type form neutral salts which are

not hydrolysed in solution and which have a relatively large first dissociation constant. They may therefore be looked upon as moderately strong acids, but their dissociation is of a peculiar type which becomes of great importance when they form one of the constituents of a buffer mixture. Ostwald has shown that the carboxyl groups in these acids affect one another in such a manner that it is only when the first stage of dissociation has progressed to a considerable extent that the second stage begins [cf. Lewis 1918, p. 267]. The peculiar behaviour of citrates may be attributed with great probability to the dissociation of the third carboxyl group suffering a steric hindrance of this nature. Thus a mixture of sodium citrate with acetic acid produces a displacement in the alkaline direction owing to the formation of a non-dissociated acid citrate.

(3) *Addition of non-electrolytes.* It is well known that when non-electrolytes are added to solutions of electrolytes the conductivity of the solution is found to decrease. This is largely due to increase of viscosity, but partly also to a decrease in the degree of dissociation of the electrolyte. Arrhenius [1892] has shown that this latter effect becomes far more marked as the degree of dissociation of the electrolyte decreases, and although the effect is somewhat complicated and depends upon the nature of both electrolyte and non-electrolyte, certain broad generalisations can be made, based upon the observations which he records.

(*a*) The effect of alcohols, ether and acetone on solutions of acids is similar, but the effect of methyl alcohol tends to be rather smaller, of acetone rather greater, than the average; the effect of cane-sugar is markedly less.

(*b*) Salt solutions show different behaviour according to type. In most cases the effect of cane-sugar is again small.

Unfortunately the effect of non-electrolytes on weak bases has not been determined.

(*c*) The influence of degree of dissociation is very marked. Arrhenius has shown that the effect of non-electrolytes upon the conductivity of acids increases with the concentration, and it is generally true that the less an electrolyte is dissociated the greater is the effect of non-electrolytes upon its degree of dissociation. There is, however, also a specific effect, as has been pointed out, the nature of which is unknown. The effect of adding non-electrolytes (which produce a decrease in the value of k) is evidently similar to that of increasing concentration (which is equivalent to a decrease in the value of v) and it is important to notice that this similarity of effect may be inferred from the symmetry of the equation

$$\alpha^2 = kv$$

in respect to these quantities, this being the form that Oswald's dilution law assumes when $1 - \alpha$ does not differ appreciably from unity.

Where k is constant we have:

$$\frac{d\alpha}{dv} = \tfrac{1}{2}\sqrt{\frac{k}{v}}.$$

Where v is constant: $\frac{da}{dk} = \frac{1}{2}\sqrt{\frac{v}{k}}$.

The rate of change of α with change of either v or k is therefore inversely as the square root of these quantities.

It is evident from what has been said above that the addition of non-electrolytes to buffer mixtures will produce a differential effect upon the constituents of these mixtures where these are unequally dissociated unless there is a compensating difference of specific effect. This differential action of non-electrolytes will show itself by a change in the reaction of a buffer solution to which alcohol or some similar substance is added. The slightly dissociated acid constituent will undergo a large reduction of its degree of dissociation, while the highly dissociated salt will be little affected; the value of $[H^{\cdot}]$ will therefore decrease. This is fully borne out by experiment.

(4) *Change of temperature.* The effect of change of temperature is difficult to predict. Noyes, Melcher, Cooper and Eastman [1910] have shown that the dissociation of neutral salts decreases very slightly with rise of temperature; salts of the type common to buffer mixtures have not been investigated, but it is probably safe to assume that it is very small. The change in the value of k is the determining factor, and though this can be calculated in certain cases the calculation usually rests on a somewhat doubtful basis.

Henderson[1] [1908] has attempted to calculate the change of reaction which takes place in a H_2CO_3–$NaHCO_3$ mixture with change of temperature, but, as he points out, the results of the calculation must be regarded as giving a not very close approximation to the true result. He however deduces from the general result the important consequence that while a small increase of $[H^{\cdot}]$ is produced in the mixture with rise of temperature, the alkalinity of the solution will rise owing to the larger temperature coefficient of dissociation of water. It is worthy of notice that this effect may possibly be greatly exaggerated in some buffer mixtures owing to the fact that the dissociation of weak electrolytes is sometimes decreased by rise of temperature.

No attempt has been made here to investigate the change of reaction which the buffer solutions of phosphates and acetates under examination experience with change of temperature. The change is probably small for most buffer solutions, but it can hardly be negligible, especially if the temperature coefficients of acid and salt should prove to be opposite in sign.

Theory of Amphoteric Electrolytes.

It is well known that solutions of ampholytes have many of the properties of buffer mixtures. Proteins are present in considerable quantities in the blood and undoubtedly help to maintain the constancy of its reaction, and it is quite probable that proteins and similar substances form a normal constituent

[1] It may perhaps be useful to point out that in Henderson's calculation of log k 38°, the number 0·491 is given in mistake for 0·481, the logarithm of 3·04. He thus obtains for k 38° the value of 4·2 instead of 4·0. The difference is probably immaterial in so approximate a result, but it leads to some confusion in his argument.

of the buffer mixtures which regulate the reaction of the protoplasm of the cell. This role must, however, be a somewhat subordinate one, and it can hardly be doubted that proteins—which may be taken as typical of colloidal ampholytes in general—have other and more important functions to fulfil in the living cell; a theory of their regulative action will be put forward later. As a preliminary it is necessary to extend this enquiry to the case of amphoteric electrolytes and to examine how these also are affected by a change of conditions.

Since ampholytes have the character of weak acids, the degree of dissociation of the acidic group which they contain will decrease as the concentration of hydrogen ions increases in the solution. This has long been recognised and it is this behaviour which is the cause of any buffer effect which they may exercise, for their capacity for forming salts with weak acids is probably very small, and ampholytes may be considered as existing in buffer solutions in the free state.

Although it is not probable that the buffer effect of ampholytes is of great general importance, it is worthy of notice that the behaviour of an acid ampholyte in respect of changes of concentration is opposite to that of the ordinary buffer solution. Walker [1904] has shown that as the concentration of an ampholyte is increased, its acidity increases, decreases, or remains constant according as k_a, the acidic dissociation constant, is less than, equal to, or greater than k_b, the basic dissociation constant. This theory of ampholytes based on the electrolytic dissociation theory was worked out for substances of the class of simple amino-acids; the application to colloidal ampholytes will be justified at a later stage. If this be assumed it is evident that the presence of proteins such as albumins or globulins in a buffer solution will tend to moderate any changes of reaction induced by changes of concentration.

It will be suggested in the sequel that the behaviour of ampholytes in the presence of non-electrolytes and at different temperatures may be a matter of very far-reaching importance. It has been shown above that $\frac{da}{dk} \propto \frac{1}{k}$; if therefore $dk_a = dk_b$ the effect of adding a non-electrolyte to the solution of an ampholyte will be to repress the dissociation of the less dissociated group relatively to that of the more dissociated and thus to remove the ampholyte further from its iso-electric point. In the immediate neighbourhood of the isoelectric point, since $d_a = d_b$, this effect will vanish.

There is however the possibility of a differential effect upon k_a and k_b, which will produce a change in the ratio k_a/k_b, therefore, k_b will be reduced in greater measure than k_a. Now Michaelis [1909][1] has shown that at the isoelectric point

$$\frac{[H^{\cdot}]}{[OH^{\prime}]} = \frac{k_a}{k_b}$$

i.e.,

$$[H^{\cdot}] = \sqrt{\frac{k_a}{k_b}} K$$

[1] For modifications of this formula see Michaelis and Rona [1919].

where K is the dissociation constant of water. A change in the ratio $\frac{k_a}{k_b}$ will therefore displace the iso-electric point.

The effect of adding a non-electrolyte to an acid ampholyte for which $k_a > k_b$ will thus be to displace the iso-electric point in the acid direction if $dk_a < dk_b$ since dk is negative.

A difference in temperature coefficients will also displace the iso-electric point, but in this case it is impossible to estimate either the magnitude or the direction of the effect.

Experimental.

The following measurements of hydrogen ion concentrations in buffer solutions have been made on the lines indicated in the preceding sections.

The apparatus used was of the ordinary type, but the usual capillary electrometer was replaced by a sensitive high resistance galvanometer. This was found to give sharp readings within 0·5 millivolt when the solutions under examination were good conductors.

The hydrogen electrodes were of Walpole's [1913] pattern; rubber stoppers were used. These electrodes, being in contact with the connecting solution, allow a slow diffusion of hydrogen through the tube of the electrode vessel. It is therefore only the surface layer that is completely saturated with hydrogen. Walpole accordingly makes contact at the meniscus. It was found more convenient in the present instance to allow the platinum point to dip just below the surface; results can be obtained by this method which agree within 1 millivolt, if a uniform procedure is adopted for saturation and sufficient time is allowed for equilibrium conditions to establish themselves. The final readings were in each case taken not less than half an hour after saturation.

The calomel electrode was of the saturated type [Michaelis and Davidoff, 1912]. This gave a reading of 0·5165 against standard acetate. Michaelis found at the temperature of experiment (25°) 0·518. The difference may be attributed to the incomplete saturation already mentioned.

The hydrogen was obtained from zinc and sulphuric acid and was purified by the usual reagents—lead nitrate, potassium permanganate and mercuric chloride. As an additional precaution it was allowed finally to bubble through $N/10$ silver nitrate, sodium hydroxide and water.

Calculations were made from the formula [Michaelis and Davidoff, 1912],

$$P_H = \frac{E - \beta}{f(t)}$$

where E is the observed E.M.F. against the calomel electrode

$$f(t) = 0{\cdot}0591 \text{ (at } 25°)$$

$$\text{and } \beta = \alpha - \gamma f(t)$$

where α = E.M.F. against standard acetate

γ = value of P_H for standard acetate = 4·62,

hence $\beta = 0{\cdot}2435$, and the formula becomes

$$P_H = \frac{E - 0{\cdot}2435}{0{\cdot}0591}.$$

All measurements were made at the temperature of 25°. Diffusion potentials were found to be very small and have been neglected. Three standard buffer solutions were used in these estimations: (1) potassium phosphate mixture ($P_H = 6{\cdot}88$) made by mixing 5 parts of $M/15$ KH_2PO_4 (P_H 4·58) with 6 parts of $M/15$ K_2HPO_4 ($P_H = 8{\cdot}38$); (2) sodium phosphate mixture ($P_H = 6{\cdot}86$) made by mixing 5 parts of $M/15$ NaH_2PO_4 ($P_H = 4{\cdot}56$) with 6 parts of $M/15$ Na_2HPO_4 ($P_H = 8{\cdot}38$); (3) sodium acetate mixture ($P_H = 4{\cdot}70$) made by mixing 5 parts of $M/5$ acetic acid with 6 parts of $M/5$ sodium acetate.

The acid phosphates which were used in the preparation of these solutions were twice re-crystallised. The solution of sodium acetate was prepared from salt obtained with the "A.R." guarantee, and once re-crystallised. As di-potassium and di-sodium phosphates are not easy to purify, the solutions of these substances were made up by mixing a weighed quantity of the acid salt with an equivalent quantity of standard hydroxide solution and diluting to the correct volume.

The neutral salts were either re-crystallised or obtained with the "A.R." guarantee, except in the case of magnesium acetate, which was tested by analysis after drying *in vacuo*, and of potassium acetate and potassium tartrate, the solutions of which were prepared by neutralising standard acid solutions until they were just alkaline to phenolphthalein. As a check on this procedure a comparison was made between solutions of $M/6$ potassium citrate prepared in this way and by solution of the salt; this gave the following results:

	P_H
By neutralising acid	6·96
From salt	6·94

Weaker solutions were generally made up by dilution from the normal. In the case of calcium and magnesium chlorides the semimolar solutions were standardised by diluting to $M/20$ and titrating with silver nitrate.

In order to carry out the comparative estimations, the results of which are shown below, the standard solutions of phosphate or acetate were diluted with their own volume either of water or salt solution. Usually 5 cc. of each were taken. In the case of the alcohols, 25 cc. of standard solution and a given volume of alcohol were made up to 50 cc. Three sets of results were thus obtained—for mixtures containing (1) potassium phosphates, (2) sodium phosphates and (3) sodium acetate and acetic acid—showing the effect of changes of concentration and addition of salts and non-electrolytes. The phosphate solutions were chosen both on account of their physiological importance and because their normal reaction is very near to absolute neutrality. The acetate solution was chosen on account of the large number of soluble acetates which allowed a more complete investigation of the action of salts.

The readings given represent in each case the mean of not less than two determinations with different hydrogen electrodes.

Table I. *Effect of Concentration.*

Vol. of water added	Potassium phosphate P_H	Sodium phosphate P_H	Sodium acetate P_H
0	6·88	6·86	4·70
1	6·96	6·96	4·71
2	7·02	7·00	4·73

The slight effect of change of concentration on the acetate mixture is to be ascribed to the fact that *M*/5 sodium acetate is already very highly dissociated; dilution can therefore produce only a slight effect.

Table II. *Effect of potassium salts of various acids upon potassium phosphate.*

	Water	Acetate *M*/4	Citrate *M*/12	Chloride *M*/4	Sulphate *M*/8	Tartrate *M*/8
P_H	6·96	6·91	6·94	6·48	6·74	6·79

Table III. *Effect of potassium citrate upon potassium phosphate.*

Concentration of salt	*M*/60	*M*/30	*M*/12	*M*/6	*M*/3
P_H	6·94	6·91	6·94	6·97	7·08

From Table II we have the series: citrate > acetate > tartrate > sulphate > chloride; but it is evident that the relative effect will vary with the concentration. In the case therefore of the other solutions the comparison has been carried over a considerable range of concentration for all the salts. The results of this comparison are shown graphically and figures are also given showing the effect of additions of metallic chlorides and of ethyl and methyl alcohol to the standard buffer mixtures. These graphs (Figs. 1–6) are self-explanatory and require little comment[1].

The addition of magnesium salts to potassium phosphate mixture produces, at concentrations above *M*/20, a labile solution which deposits magnesium phosphate on standing. A large rise of $[H^{\cdot}]$ is to be observed in the solution when this has taken place, but some of the previous effect must also be due to the formation of undissociated magnesium phosphate. A comparison of the effects of magnesium chloride and magnesium acetate shows that in concentrated solution the nature of the acid which replaces phosphoric acid largely determines the effect.

The alcohol measurements confirm the theoretical deduction of a decrease of $[H^{\cdot}]$ in the presence of non-electrolytes. In the case of the alcohols this is very marked. It was hoped to carry out similar observations on mixtures containing sugar, but the present apparatus proved quite unsuitable for the treatment of highly viscous solutions, and the observations had therefore to be abandoned. There is however very little doubt, in the light of Arrhenius' work, that these mixtures would show a similar decrease of hydrogen ion concentration, but that the effect would be much less marked.

[1] The concentrations of salt solutions in these figures are expressed in terms of normality. By a normal solution is here meant a solution containing one equivalent of base per litre, *i.e.* *M*/3 sodium citrate, *M*/2 calcium chloride, etc.

Physiological Effects.

The effect of buffer solutions and ampholytes in determining reaction has been demonstrated in the preceding sections, and some consideration must now be given to the physiological consequences which may ensue when these changes take place in the living cell. The whole trend of modern physiology is to emphasise the preponderant importance of the reaction of the medium, in determining the rate and direction of the processes of metabolism, but the processes of metabolism which take place in living protoplasm are themselves processes of chemical change which tend to modify reaction, *e.g.* by

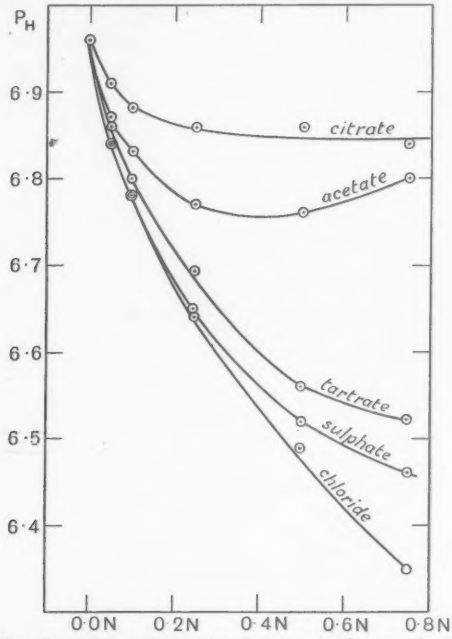

Fig. 1. The effect of the sodium salts of various acids upon the reaction of sodium phosphate mixture.

production of carbon dioxide and changes in the concentration of salts, and non-electrolytes in the cell may also produce changes in the reaction of the buffer solutions of the protoplasm. A regulative process is thus at work which it will be necessary to examine more closely.

Before proceeding to more definitely physiological considerations, it is desired to lay some stress upon the action of the anions of the neutral salts. It has been shown that the salts of various acids differ in the effect which they produce upon the reaction of buffer mixtures; consequently we have here an anionic series similar to the well-known Hofmeister series and to those obtained in various physiological reactions. It is to be noticed that the order

of the series of anions expressing the effect of salts upon the reaction of buffer mixtures varies to some extent with the nature of the buffer mixture and also with the concentration at which the salts are compared.

It has usually been the custom in physiological and biochemical work to make comparison between the molar concentrations necessary to produce a given effect. In the graphs (Figs. 1–4) equivalent concentrations have been compared. It is interesting to notice that the Hofmeister series falls (with

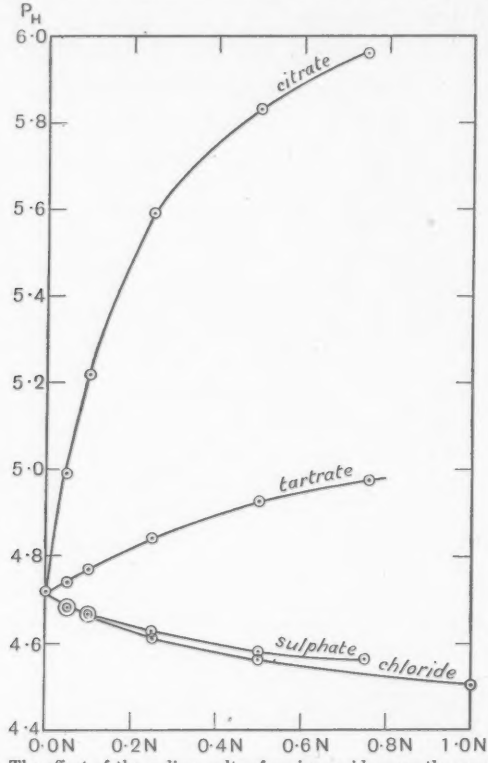

Fig. 2. The effect of the sodium salts of various acids upon the reaction of sodium acetate mixture.

the exception of chloride) into two well-defined groups, if equivalents rather than molecular concentrations are compared. Thus:

Concentration of salt necessary for the precipitation of egg albumin.

	Mols.	Equivalents
Citrate	0·56	1·68
Tartrate	0·78	1·56
Sulphate	0·80	1·60
Acetate	1·69	1·69
Chloride	3·62	3·62
Nitrate	5·42	5·42
Chlorate	5·52	5·52

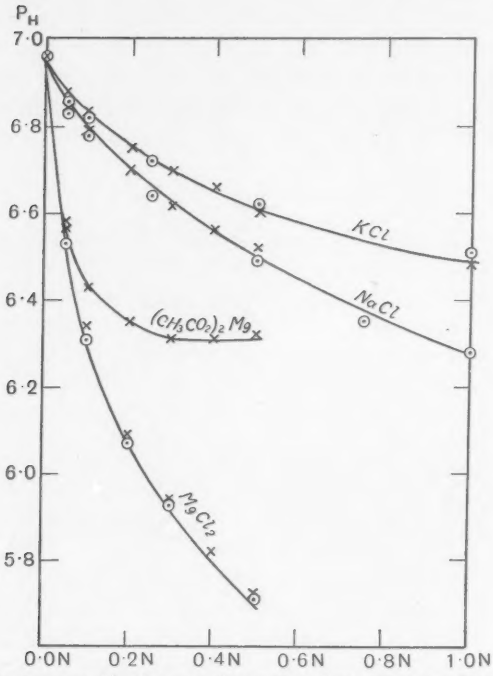

Fig. 3. The effect of various chlorides upon the reaction of potassium phosphate mixture (×) and sodium phosphate mixture (⊙) and of magnesium acetate upon potassium phosphate mixture.

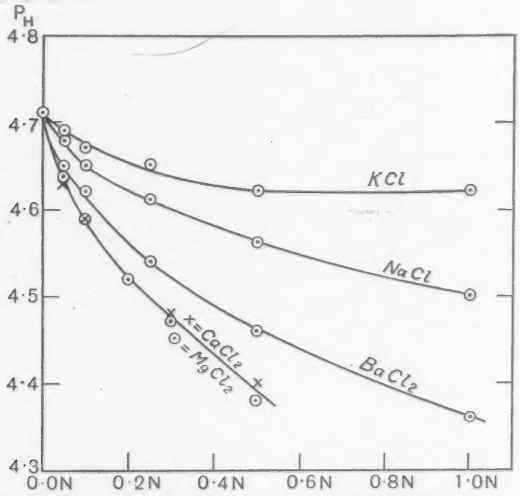

Fig. 4. The effect of various chlorides upon the reaction of sodium acetate mixture.

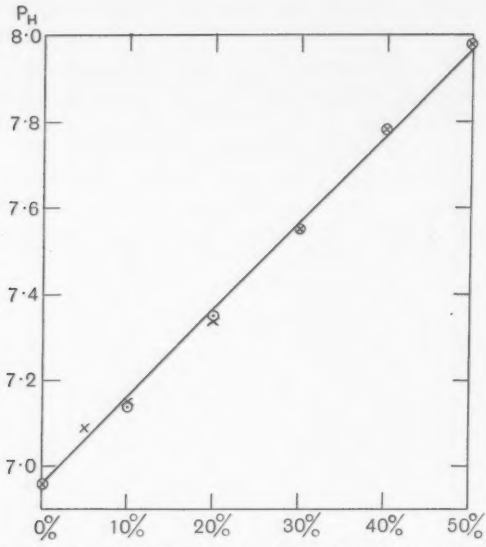

Fig. 5. The effect of methyl alcohol (×) and ethyl alcohol (⊙) on the reaction of potassium phosphate mixture.

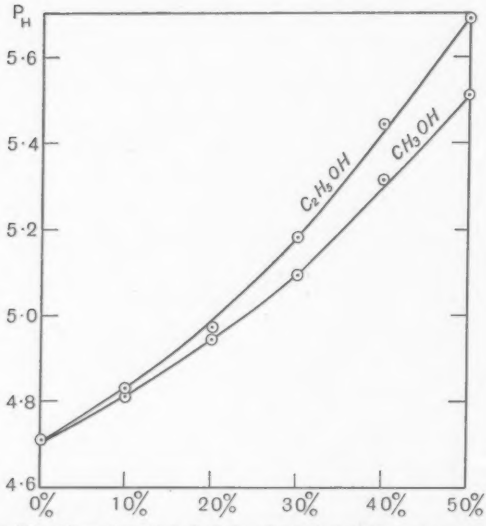

Fig. 6. The effect of methyl alcohol and ethyl alcohol on the reaction of sodium acetate mixture.

It is probable that neither in physiological nor in biochemical work has sufficient attention been given to the effects of changes of reaction arising from the double decomposition of substances of weakly acidic nature, which form ions of the type HX' or undissociated molecules and thus immobilise hydrogen ions.

Theory of Permeability.

It has been shown above that the effect of mineral salts upon buffer solutions is to increase acidity, and that salts having bivalent cations are far more active in this respect than those whose cations are univalent. These chemical differences, together with the small but definite differences of behaviour which are shown by similar salts of sodium and potassium, are clearly of importance as indicating a possible explanation of such physiological differences as are exhibited in the phenomena of "the antagonism of ions." Loeb has attempted to explain antagonism as a consequence of the colloidal properties of the cell and more particularly of the plasma membrane. He applies the rules worked out by Hardy and Schulze for the precipitation of suspensoid colloids, and regards the action of salts upon the living cell as effecting an incipient coagulation of the plasma membrane [see Höber 1914, pp. 529, 530]. There are however considerable difficulties in accepting this explanation as it stands, especially as it is doubtful whether the precipitating action of salts upon suspensoids and emulsoids is due to similar causes. Moreover Loeb's theory gives no explanation of the differences of behaviour which are shown by ions of equal valence. If, however, as is now suggested, buffer solutions are called in to play a part in these phenomena, the range of possible explanation becomes greatly widened. The normal reversible effects of salts upon protoplasm may then be looked upon as indirect and due to modifications in the reaction of the fluid bathing the protoplasm, while direct action upon the proteins or similar substances of the protoplasm itself will produce abnormal and usually irreversible effects. These considerations are of especial importance in relation to the plasma membrane, and it is proposed to consider in some detail the effect which changes in the reaction of the fluid medium may produce upon the proteins of the membrane, and to suggest a theory of permeability based upon these changes. The main outlines of this theory are as follow:

(1) The semi-permeable membrane is assumed to be a gel; the more solid phase of which consists principally of emulsoid colloids having an amphoteric character, while the liquid phase is of the nature of a buffer mixture.

(2) There is maximum permeability at the iso-electric point. Permeability to salts falls off very rapidly on either side of the iso-electric point.

A few words are necessary in reference to these assumptions.

There has been a considerable change of opinion in recent years as to the nature of emulsoid solutions, but most chemists are now agreed in regarding them as partaking very largely of the nature of true solutions from which

they differ chiefly in virtue of the size and complexity of their particles [see Höber 1914, p. 304; Sörensen 1917, p. 4].

The recent work of McBain [1920] has shown that these particles are usually aggregates of both ions and non-dissociated molecules which contain large quantities of water, and that their composition "varies continuously with changes in concentration and temperature or upon the addition of salts." There is also much evidence that the hydration of the particles is a function of their charge; indeed the varying viscosity of protein solutions has long been interpreted as due to variations in the number of the heavily hydrated ions into which protein dissociates. This conception of "micellae[1]" capable of undergoing continuous changes of charge and water content is evidently of great value to the biologist in enabling him to form some picture of the changes which protoplasm may undergo, and in particular of the variations which may take place in the state of aggregation of the plasma membrane of the cell.

The theory of protein dissociation and of the iso-electric point in relation to ampholytic colloids has been the subject of much controversy and there is little doubt that the dissociation of proteins differs considerably from that of the simple amino-acids. There is however much evidence that proteins are ampholytes whose acidic and basic characters depend upon the dissociation of hydrogen and hydroxyl as do those of other weak acids and bases, and that their dissociation is at a mimimum at the iso-electric point[2]. Sörensen, Höyrup, Hempel and Palitzsch [1917] have recently measured the iso-electric point of highly-purified egg albumin in solutions containing sulphuric acid and ammonium sulphate, using the principle of Michaelis[3], that if an ampholyte is added to a solution the P_H of which is that of the ampholyte at its iso-electric point the reaction of the solution will be unchanged. They obtain a constant value for the iso-electric point of egg albumin under very varying conditions, thus affording strong support to Michaelis' theory of the iso-electric point.

Little direct evidence exists as to the chemical nature of the membrane, but there is a very strong probability that proteins are present in it and there is also much reason to believe that it contains lipoid substances. It is therefore legitimate to assume that it consists largely of ampholytic colloids. The properties of ampholytic colloids have been principally studied in the case of proteins, and although lecithin is known to possess an ampholytic character, no detailed investigation of its behaviour has been made. It will therefore be necessary, pending the results of further investigation, to assume the properties of protein as typical of the ampholytes of the semi-permeable membrane.

[1] It seems convenient to follow the current botanical use and to write micellae as the English plural of Nägeli's term.

[2] A very complete summary of the evidence relating to the dissociation of proteins and the theory of the iso-electric point is given by Robertson [1918] and Pauli [1914, 1920].

[3] For a recent amplification of Michaelis' theory see Michaelis and Rona [1919] in which references to the earlier developments of the theory are given.

It has been shown that the plasma membrane may be regarded as composed of particles undergoing continuous changes of charge and water content according to variations in the reaction of the surrounding medium. We have now to consider the conditions for permeability in such a membrane. Substances soluble in the continuous phase of the gel—lipoid-soluble substances—will obviously pass through with more or less ease in all circumstances and will experience no regulative action. Water-soluble substances on the contrary will have to pass through pores, the size of which will vary with the state of hydration of the colloidal particles, and will therefore pass through most easily when those pores are at their maximum size, *i.e.* at the iso-electric point. It is moreover to be expected that this variation in permeability will be greatly exaggerated in the case of charged ions; at reactions above that of the iso-electric point the membrane will carry a positive charge and will repel cations; at reactions below this point it will carry a negative charge and repel anions; in each case it will also impede the movement of the oppositely charged ion, on which it will exercise an attraction. It is only in the immediate neighbourhood of the iso-electric point that the membrane will be permeable to ionised salts as a whole. A membrane of this nature will thus possess a variable permeability, especially to electrolytes, and will exercise a regulative function conditioned largely by changes in the reaction of the plasma medium, since by these changes the ampholytes of the membrane will necessarily be brought nearer to the iso-electric point or will be removed further from it. It is clear therefore that neither salts nor non-electrolytes can be without effect upon its permeability, and this effect must now be considered in detail.

To begin with the case of non-electrolytes. It has been shown above that these reduce the acidity of a buffer mixture and also that there is strong reason to suppose that if they are added to the solution of an ampholyte they may displace the position of the iso-electric point. Non-electrolytes may therefore affect permeability in two different ways; action on the buffer solutions of the membrane will take place in the liquid phase, action on its ampholytes in the solid phase, but one effect will usually be negligible since most substances are relatively insoluble in one of the two phases of the membrane.

For water-soluble substances such as sugar the principal effect will be on the buffer solutions of the liquid phase, while lipoid-soluble substances such as ether, which are largely soluble in the solid phase of the membrane, will act chiefly by displacing its iso-electric point. Alcohol it may be noticed will be soluble in both phases. It will be convenient to leave the further consideration of the action of the lipoid-soluble substances to a later stage and to deal with them under the head of narcotics. Most food materials are water-soluble; the only important exception is fat, and the fats are readily hydrolysable into water-soluble substances.

It has been shown that there is much reason to suppose that a change in the concentration of sugar in the protoplasm of the cell will change the

reaction of the buffer mixtures of the protoplasm and thus bring it nearer to or remove it further from the iso-electric point. If the protoplasm is below the iso-electric point a decrease in the concentration of sugar will bring it nearer to this point and thus promote permeability and diffusion; if the protoplasm is above the iso-electric point, an increase of concentration will have the same effect[1]. As, however, the effect of the sugars upon dissociation is small, it may be expected that considerable changes of concentration will be necessary to produce any marked physiological effect.

The diffusion of salts into the cell is conditioned by two factors—the concentration of the salt and the change of reaction which it produces. If the reaction of the buffer mixture of the plasma membrane is above its iso-electric point, the diffusion of salt into the cell will increase acidity, and thus an increasing resistance to the entrance of more salt will be produced. It is here that the antagonism of ions will come into play. Calcium chloride will cut down permeability at a much lower concentration than sodium chloride; therefore the addition of a little calcium chloride to a solution of sodium chloride will impede its penetration. On this hypothesis it is easy to understand such observations as those of Osterhout [1911] and Loeb [1916]. Osterhout showed that solutions of sodium chloride and calcium chloride, neither of which was capable of plasmolysing the cells of Spirogyra, effected rapid plasmolysis when mixed. Loeb found that the amount of calcium chloride necessary to prevent injury to eggs of *Fundulus* by sodium chloride solution varied with the concentration of the sodium salt. The results obtained by Stiles and Kidd [1919] are also significant. They found that the rate of absorption fell rapidly after a few hours, and that though calcium was rapidly absorbed at first, at final equilibrium bivalent ions were absorbed to a much less extent than univalent. Another important point in this connection is the antagonism between ions of a like valency, *e.g.* sodium and potassium, calcium and magnesium. It seems probable that this also can be explained by the different effect which they produce on the reaction of the medium. It has been observed that both phosphate and acetate mixtures show a definite difference of response to potassium and sodium salts; the effect of calcium and magnesium could be compared only on sodium acetate mixture, and in this case their reactions were alike, but since the chemical differences between the salts of calcium and magnesium are frequently considerable, this case cannot be general and it is probable that the antagonism may find a general explanation in differences in the properties of the products of reaction of the salts in question with the constituents of the buffer mixture of the protoplasm. It should however be remarked that the striking physiological effect of very small traces of certain substances appears to indicate a direct action upon the plasma membrane in some cases. The foregoing considerations show clearly the necessity for main-

[1] The excretion of sugar from plant cells when the concentration of sugar becomes high is thus explained. It is suggested below that in leaves the plasma membrane is normally maintained above the iso-electric point.

taining a definite ratio in the concentration of the various ions present, since any modification will affect the reaction of the protoplasm. The "balanced" nature of such mixtures as Ringer's solutions, sea-water, and the water-culture solutions used for the growing of plants is therefore explained.

No definite hypothesis has hitherto been made in this paper as to the reaction of the protoplasm and the plasma membrane in respect to the iso-electric point. Electrical phenomena which will be discussed later suggest that the membrane of many cells is normally permeable to cations. This indicates that the plasma membrane of such cells is below the iso-electric point.

In the case of leaves, changes in the concentration of carbon dioxide due to alteration of the balance between respiration and assimilation must lead to changes of permeability, and a variation in the supply of salts would produce a like effect. If the reaction is above the iso-electric point, permeability and diffusion will be at a maximum during periods of active photosynthesis. This is in accordance with the observations of Lepeschkin [1909], Tröndle [1911], and Blackman and Paine [1918], who all found a marked increase of permeability as the result of increased illumination. It is possible too that a falling off in the supply of salts is one of the causes of the death of leaves, since death is known to be preceded by increased permeability.

Equilibrium in the cell. It is well known that the living cell can maintain permanent differences of concentration on either side of a membrane which it is impossible to regard as completely impermeable (cf. Abderhalden's analyses of blood corpuscles and serum [1909]; also Stiles and Kidd [1919]). The question of equilibrium is very complex and a number of factors are involved, of which permeability may not in many cases be the most important. It seems probable however that there is true dynamic equilibrium in certain cases, and this must be due to the properties of the membrane. As an example reference may be made to the exclusion of organic acids from the protoplasm by the action of the vacuole membrane, such as is known to occur in the leaf of Oxalis. When the cell sap is highly acid it is probable that the vacuole membrane is maintained at a reaction far above that of its iso-electric point. In this case it is probably impermeable to ions of either sign. Equilibrium may thus be maintained between the non-dissociated fraction on either side of the membrane. Overton [1902] has shown that the plasma membrane is in general in some degree permeable to undissociated molecules which thus behave like any other non-electrolyte, and it may be supposed that the membrane of the vacuole has similar properties. Any acid present in the vacuole will of course be dissociated on passing the membrane, but in presence of its salts the hydrogen ion concentration will be kept very low. We have thus the possibility of equilibrium between acid present in the vacuole and in the protoplasm, while at the same time the hydrogen ion concentration of the latter is at a minimum. In the case of permanent differences of salt concentration, such as are found between blood corpuscles and serum, it may be objected

that such an equilibrium could not be maintained, since salts are only completely dissociated at infinite dilution. This is of course true, if an undissociated fraction is really present, but the existence of strong electrolytes in the undissociated state has recently been called in question. Ghosh [1918] has found that the abnormalities of strong electrolytes can be satisfactorily explained on the supposition that they are completely dissociated at all concentrations.

The question of differential absorption, as *e.g.* by such an organ as the root, is closely related to that of equilibrium, and here again a number of factors are probably concerned; it is nevertheless probable that a true differential permeability exists, of which the difference between sodium and potassium ions affords the most striking instance. Potassium ions are highly diffusible; whether this is to be ascribed to a low degree of hydration, to a small atomic volume, or to any other difference of physical conditions, is at present undetermined (see *Faraday Soc. Trans.* [1919]), but on almost any hypothesis potassium salts will continue to penetrate the membrane in virtue of their diffusibility, under conditions which completely exclude sodium salts. Moreover, the effect of potassium ions upon the reaction of buffer mixtures is small and increases slowly with increasing concentration; they may therefore reach a considerable concentration before being also in their turn cut off by the lessened permeability of the membrane.

Stimulation. There is very little doubt that stimulation is accompanied by changes of permeability. It is therefore necessary that any theory of the plasma membrane should take account of the phenomena of stimulation. These phenomena have chiefly been studied on nerves or muscles. It will be assumed here that the protoplasm in such tissues is below the iso-electric point, in which case the membrane will be permeable to cations. In this case a slight excess of hydrogen ion concentration within the cell will produce a diffusion potential and the current of injury can be explained on Bernstein's hypothesis as due to a migration of anions from the interior of the cell at the point where the membrane is pierced. The current of rest is susceptible of an explanation on similar lines, for salts will produce an increase of hydrogen ion concentration at the place at which they act and will bring the membrane nearer to the iso-electric point—the point of permeability.

It is not proposed to consider the general theory of stimulation here; it may however be pointed out that on Nernst's hypothesis stimulation is accompanied by an increase in the concentration of salt in the immediate neighbourhood of a membrane: on the present hypothesis the production of increased permeability follows as a necessary consequence.

Narcosis. If stimulation is to be explained by the production of permeability at a point of the membrane, it is reasonable to suppose that the effect of narcotics is to reduce permeability. This explanation accords well with observations on the effect of narcotics on the production and transmission of excitation, and there is some direct experimental evidence in its favour; but direct experiments on permeability present very special difficulties both in

execution and interpretation, and the results cannot be considered conclusive (for a critical review of the evidence see Katz [1918]). The general result of different lines of enquiry is however to confirm the conclusion that true narcosis is the result of a decrease of permeability of the membrane, and that increase of permeability is only produced by narcotics at relatively high con centrations in which case the effects are irreversible and injurious.

It has been shown that the alcohols produce a decrease of hydrogen ion concentration in buffer solutions, and that their action is thus the reverse of that of salts. This accords well with the theory that narcotics act by reducing permeability. Alcohols cannot however be taken as typical of narcotics in general, for these are normally very slightly soluble in water. Such substances act directly on the solid phase of the membrane, and as already pointed out there is reason to suppose that their action may consist in a displacement of the iso-electric point. If so narcotics may be regarded as lipoid-soluble substances which by their differential effect on the dissociation constants of the ampholytes of the membrane remove them further from their iso-electric condition.

A few words remain to be said in conclusion. No attention has been given to the possible effects of adsorption, but it is very probable that buffer solutions are adsorbed by the membrane, and it is not unlikely that excitation may in some cases be due to changes in the concentration of these salts produced by changes of surface tension. Theories relating to tropisms have also been left unconsidered, but it may be worth while to remark that on the present hypothesis the relation of the membrane to the iso-electric point will determine whether the reaction to the tropic stimulus is positive or negative. Small [1920] has already made the suggestion from a different point of view that the tissues of the root may be acid and those of the stem alkaline.

It is fully recognised that the experimental basis of certain of the theories which have been put forward in the present paper is very incomplete. It is hoped in the future to carry out experiments on the effect of non-electrolytes and of temperature on the iso-electric point of amphoteric electrolytes. It is also intended to study the specific effect of non-electrolytes on dissociation. In the meantime it is desired to point out that the present theories relating to permeability are based very largely on hypotheses which have been found necessary in the study of other phenomena. The theory of the buffer solutions present in protoplasm, of the existence of protein and lipoid substances in the plasma membrane, and of their ampholytic character, are all well established on other grounds, and the present theories consist almost entirely of necessary deductions from these assumptions. That it is possible by their means, not only to explain differences of permeability, but to correlate such well-established physiological facts as the antagonism of ions and the various phenomena of electric stimulation and the effect of narcotics, is strong evidence in their favour, especially in view of the fact that they show how the regulation of permeability can be a direct result of cell metabolism.

SUMMARY.

The theory of buffer solutions shows that an increase of hydrogen ion concentration normally results from the addition of neutral salts, a decrease from the addition of non-electrolytes. These deductions have been confirmed by experiments on buffer mixtures of phosphates and acetates.

There is a marked difference of effect between univalent and bivalent cations, and a small but distinct difference has been observed between sodium and potassium salts.

It is shown on theoretical grounds that the addition of non-electrolytes to the solution of an ampholyte may displace its iso-electric point. In the case of an ampholyte for which $dk_a < dk_b$, the iso-electric point will be displaced in the acid direction, since dk is negative.

A theory of permeability, based on the foregoing observations and deductions, is suggested. The semi-permeable membrane is assumed to be a gel, the more solid phase of which consists principally of emulsoid colloids having an amphoteric character, the liquid phase of a buffer mixture. Such a membrane will have a maximum permeability at the iso-electric point, since at this point the continuous phase will not only be in a state of minimum hydration and will thus occupy a minimum volume, but will also be without charge. Above the iso-electric point the membrane will carry a positive charge and will repel cations; below this point it will carry a negative charge and repel anions. The membrane will therefore be peculiarly impermeable to charged ions, except in the immediate neighbourhood of the iso-electric point. Additions of salts and non-electrolytes will modify the permeability of the plasma membrane by the changes which they produce in the reaction of the buffer mixture of the protoplasm.

The phenomena of antagonism can be explained as resulting from the different effect of different cations on the reactions of the buffer mixture of the protoplasm.

The bearing of the present theory upon stimulation and narcosis is outlined.

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REFERENCES.

Abderhalden (1909). Lehrb. physiol. Chem.
Arrhenius (1892). *Zeitsch. physikal. Chem.* **9**, 487.
Bayliss (1915). Principles of General Physiology.
Blackman and Paine (1918). *Annals of Botany*, **32**, 69.
Faraday Soc. Trans. (1919). **15**, 1.
Ghosh (1918). *J. Chem. Soc.* **113**, 449, 627, 707, 790.
Hempel (1917) *Compt. rend. Carlsberg*, **13**, 1.
Henderson (1908). *Amer. J. Physiol.* **21**, 173, 427.
Höber (1914). Physik. Chem. d. Zelle.
Katz (1918). *Biochem. Zeitsch.* **90**, 153.
Loeb (1916). The Organism as a Whole.
Lepeschkin (1909). *Beitr. Bot. Centralbl.* **24**, 308.
Lewis (1918). System of Physical Chemistry.
McBain (1920). *Proc. Royal Soc.* A, 1920, **97**, 44. Third Report on Colloid Chemistry—Dpt. of Scientific and Industrial Research, 2.
Michaelis (1909). *Biochem. Zeitsch.* **19**, 181.
Michaelis and Davidoff (1912). *Biochem. Zeitsch.* **46**, 148.
Michaelis and Rona (1919). *Biochem. Zeitsch.* **94**, 225.
Noyes, Melcher, Coopèr and Eastman (1910). *Zeitsch. physikal. Chem.* **70**, 335.
Osterhout (1911). *Science*, **34**, 187.
Overton (1902). *Arch. ges. Physiol.* **92**, 115.
Pauli (1920). Kolloidchemie der Eiweisskörper.
Pauli, Samec and Strauss (1914). *Biochem. Zeitsch.* **59**, 470.
Pauli and Handovsky (1909). *Biochem. Zeitsch.* **18**, 340.
Robertson (1918). The Phys. Chem. of the Proteins.
Small (1920). *The New Phytologist*, **19**, 49.
Sörensen (1917). *Compt. rend. Carlsberg*, **12**, 1.
Sörensen, Höyrup, Hempel and Palitzsch (1917). *Compt. rend. Carlsberg*, **12**, 68.
Stiles and Kidd (1919). *Proc. Roy. Soc.* B, **90**, 448, 487.
Tröndle (1911). *Jahrb. Bot.* **48**, 171.
Walker (1904). *Proc. Roy. Soc.* **73**, 155; **74**, 271.
Walpole (1913). *Biochem. J.* **7**, 410.